Drosophila *E2F* and *DP* genes: their role in the regulation of G1-S progression and the activity of DNA replication origins

by

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ABSTRACT

Late in Drosophila embryogenesis the cells giving rise to most of the larval tissues switch from a mitotic cycle to the endo cycle. The endo cycle consists of only S phase and a gap phase, leading to polyteny. It is preceded by the first detectable G1 period in Drosophila embryogenesis and developmentally regulated induction of S phase genes in the pattern of DNA synthesis. My analysis of the regulation of G1-S progression began with a genetic screen for mutations that disrupt the transcription pattern of S phase genes in mitotic and polytene cells of the embryo. A number of mutations in known and novel genes were isolated. Further analysis and phenotypic characterization has focused on the mutations that nearly eliminate the transcription of S phase genes in mitotic and polytene cells. These mutations are in the Drosophila homologs to the mammalian *E2F* and *DP* genes. The E2F transcription factor, a heterodimer of E2F and DP subunits, is linked to the G1-S transition in mammalian cells. Five mutations in the Drosophila *DP* gene (*dDP*) were isolated and provide the first opportunity to examine the *in vivo* role of this gene. Despite a pronounced effect on the G1-S transcription of S phase genes in *dDP* and *dE2F* mutant embryos, a block to replication was not observed. Null mutations in *dDP* and *dE2F* cause lethality late in development with some mitotic and polytene tissues being underdeveloped or absent. The mutant phenotypes reveal a positive role for E2F/DP in cell cycle progression in mitotic and endo cycle cells. Weak alleles of *dE2F* and *dDP* develop to adulthood and exhibit defects in oogenesis. Analyses of these mutants showed that E2F/DP controls differential regulation of replication origins within polyploid S phase. In ovarian nurse cells, E2F/DP limits replication of heterochromatic sequences. In follicle cells, E2F/DP is required to shut off genomic replication and activate the amplification of chorion loci. In addition to the positive and negative effects on the activity of replication origins, E2F/DP is necessary for nuclear lamin breakdown in nurse cells and subsequent nurse cell apoptosis.

Thesis Supervisor: Terry L. Orr-Weaver
Title: Professor of Biology

To the memory of Shlema-Zelman Royzman
and Sonia Perlmutter

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TABLE OF CONTENTS

Chapter One

Introduction

The cell cycle, the ordered sequence of events by which cells grow and divide, has been studied in diverse organisms including yeasts, frogs, flies, and mammals. A typical cell cycle consists of a round of DNA replication (S phase) and subsequent chromosome segregation and cell division (M phase); a G1 gap phase separates the end of M phase and the beginning of S phase, and a G2 gap phase defines the period from the end of DNA synthesis to the beginning of the next M phase. S phase and mitosis alternate with each other to ensure the proper genome ploidy and viability of daughter cells.

The ability to deviate from the typical cell cycle order, however, is key for a number of developmental processes. The cell cycle resulting in the production of haploid gametes, meiosis, requires two rounds of M phase without an intervening S phase. Many cells become polyploid or polytene. These cells tend to be highly metabolically active and synthesize large amounts of particular products in a short period of time. The trophoblast cells that give rise to the mammalian placenta, and ovarian nurse and follicle cells critical for Drosophila oocyte development are examples. In fact, 300 examples of polyploidy or polyteny have been observed in animals, plants, and protozoans (Brodsky and Uryvaeva 1984; Nagl 1978). It has been demonstrated in Drosophila that polyploid and polytene cells result from an altered cell cycle in which S phase alternates with gap phase (Hammond and Laird 1985a; Hammond and Laird 1985b; Mahowald, et al. 1979; Pearson 1974; Rudkin 1973; Smith and Orr-Weaver 1991). This modified cell cycle, termed the endo cycle, excludes cell division. Thus, growth results from an increase in cell size rather than cell number, and increasing polytenization is the primary mechanism of larval growth in Drosophila. In addition, 20-30% of the DNA is under-represented in polytene and polyploid cells due to incomplete replication of late-replicating regions of the genome (Hammond and Laird 1985a; Lilly and Spradling 1996; Rudkin 1969). This change in

the parameters of S phase begins with the first endo cycle of Drosophila embryogenesis (Smith and Orr-Weaver 1991).

To isolate S phase controls that either link or distinguish mitotic and polytene cells, I screened for mutations that disrupt G1-S transcription late in Drosophila embryogenesis. The mutations that were recovered from this screen are discussed in Chapter 2. This thesis focuses on the shared regulators of the mitotic and endo cell cycles, the Drosophila *dDP* and *dE2F* genes. The E2F transcription factor, a heterodimer of E2F and DP subunits, is linked to the G1-S transition in mammalian cells. In Chapter 3, the effects of null mutations in *dDP* and *dE2F* on G1-S progression in mitotic and polytene tissues are examined. The analysis of weak, female-sterile *dDP* and *dE2F* mutations is presented in Chapter 4; this study links E2F to the differential use of replication origins within polyploid S phase and to developmentally-induced apoptosis.

This introductory chapter is divided into three parts. The first part examines the relationship between the mitotic and endo cell cycle. I discuss the various mutants and treatments that have provided insights into the molecular mechanism responsible for the bypass of mitosis in polytene and polyploid cells. Next, I provide an overview of the G1/S transition in budding yeast, mammalian cells and flies. Finally, Drosophila oogenesis is presented as a model system for the study of differential replication.

Breaking the order of S and M phase

In a typical cell cycle, the onset of S phase depends on the completion of mitosis. This dependence was illustrated in a series of elegant mammalian cell fusion experiments. These studies showed that when an M phase cell is fused with a cell in any other part of the cell cycle, hybrid cells are produced in which all the nuclei enter mitosis (Johnson and Rao 1970). Fusing an S-phase cell with a G1 cell, induces the G1 nucleus to replicate its DNA prematurely. Despite the presence of a dominant inducer of DNA replication in S phase cytoplasm, the fusion of a G2 cell with an S phase cell does not permit the G2 nucleus to replicate its DNA (Rao and Johnson 1970). Thus, entry into S phase requires the absence of a dominant inducer of mitosis, removal of the block to replication, and an S phase activator.

The dominant inducer of mitosis is MPF, a complex of protein kinase $p34^{cdc2}$ and mitotic cyclin B. MPF has been highly conserved during evolution, and its universal importance to the regulation of M-phase in eukaryotic cells is established (Nurse 1990). Mitosis is induced due to accumulation of cyclin B during interphase, and the subsequent degradation of cyclin B inactivates MPF and resets the cell cycle (Minshull, et al. 1989; Murray and Kirschner 1989; Murray, et al. 1989). In addition, it has been suggested that mitosis allows an activator of S phase to gain access to DNA upon nuclear envelope breakdown. A G2 nucleus made permeable with a membrane disrupting agent and then repaired can undergo rereplication of the DNA replication in Xenopus egg extracts (Blow and Laskey 1988).

Mutants in fission yeast suggest that $p34^{cdc2}$-mitotic cyclin B complex determines the order of S and M phase in the eukaryotic cell cycle (Nurse 1994). The *cdc2* gene (encoding $p34^{cdc2}$) is required in G1 at START when the cell becomes committed to the cell cycle as well as at M phase (Nurse and Bissett 1981). Heat treatment of specific temperature-sensitive *cdc2* mutations result in DNA replication without an intervening mitosis: destruction of $p34^{cdc2}$ before the onset of M phase

resets the cell cycle, and on restoration of $p34^{cdc2}$ function at the permissive temperature the mutants initiate a second round of S phase instead of undergoing mitosis (Broek, et al. 1991). Also, cells deleted for *cdc13* (encoding mitotic cyclin B) undergo repeated rounds of S phase in the absence of mitosis, and overexpression of *cdc2* and *cdc13* in G1 induces premature entry into M phase (Hayles 1994). These results suggest that the $p34^{cdc2}$-mitotic cyclin B complex identifies a cell as being in G2/M, and prevents DNA replication; the absence of this complex in G2 cells after heat treatment of $cdc2^{ts}$ or elimination of cyclin B is sufficient to overcome the block to replication. Normally, mitotic cyclin B is degraded at the end of mitosis, and the cell cycle is reset to G1. Thus, there is a remarkable convergence between the phenomena revealed by the cell fusion experiments, frog biochemistry and yeast genetics.

A number of other mutants and treatments also suggest that the mitotic program inhibits S phase. The fission yeast *rum1* gene (encoding an inhibitor of $p34^{cdc2}$) when overexpressed blocks M phase and allows repeated S phases (Moreno and Nurse 1994). It is known that protein kinase inhibitors can induce S phase without mitosis in mammalian cells (Usui, et al. 1991). Loss of ts41 function in Chinese hamster cell lines (at the nonpermissive temperature) also blocks M phase and results in polyploid cells. The wild-type *ts41* gene product is proposed to be essential for mitosis, and its activity may repress S phase (Handeli and Weintraub 1992).

It seems likely that meiotic cells use cdc2/cyclin B activity to prevent DNA replication from intervening between the two meiotic divisions. In Xenopus oocytes, cyclin B persists between the meiotic divisions (Kobayashi, et al. 1991), and the levels of MPF remain high (Furuno, et al. 1994; Ohsumi, et al. 1994). The high level of MPF may suppress S Phase. This is consistent with the observation in Xenopus embryo extracts that the assembly of replication proteins on DNA is inhibited by the

$p34^{cdc2}$-mitotic cyclin B complex (Adachi and Laemmli 1994). Furthermore, expression of a dominant negative cdc2 or inhibition of Mos in oocytes causes replication between the meiotic divisions (Furuno, et al. 1994). Mos is needed to maintain cdc2/cyclin B active. Thus, in both situations the inappropriate round of S phase may result from a failed cdc2/cyclin B activity.

In contrast to meiotic cells, cells that become polytene or polyploid during the normal course of development shut off mitotic functions. Indeed, Drosophila endo cycles do not require *cdc2* (Reed and Orr-Weaver 1997; Stern, et al. 1993), and mitotic functions appear to be shut off in polytene and polyploid cells. Mitotic cyclin B has not been detected in endo cycle tissues (Lehner and O'Farrell 1990; Lilly and Spradling 1996). Moreover, mutations in *cdc2* cause normally diploid tissues to become polytene, suggesting that the downregulation of cdc2/cyclin B may be sufficient to drive repeated rounds of S phase (Hayashi 1996).

It is possible that the mechanism responsible for repressing mitosis in polytene and polyploid cells involves novel, endo cycle specific regulators. However, endo cycle cells may utilize the same cell cycle controls that permit mitotic cells to end mitosis and reset the cell cycle. The effects resulting from mutations in the Drosophila gene *morula* support the latter mechanism (Reed and Orr-Weaver 1997). Nurse cells of the adult ovary become polyploid; mitotic spindles, condensed chromosomes, and mitotic cyclin B are never observed. In female-sterile *morula* mutants nurse cells initiate endo cycles, but then revert to mitosis; the nuclei contain large mitotic spindles, metaphase-like chromosomes, and cyclin B. The inappropriate presence of cyclin B in mutant nurse cells suggests that the activity of the cdc2/cyclin B kinase may be high. Mitotic cells require degradation of the cyclin subunit of the cdc2/cyclin B kinase for passage though the metaphase/anaphase transition (for review see King, et al. 1996). In the lethal *morula* alleles mitotic cells are blocked at metaphase, suggesting that the cdc2/cyclin B kinase remains active

(Reed and Orr-Weaver 1997). Thus, morula serves a dual function: it promotes exit from mitosis in diploid cells and maintains the shut off of mitotic activities in polyploid cells.

A recent study suggests that the anaphase-promoting complex (APC) is up-regulated in polyploidizing megakaryocytic cell lines (Zhang, et al. 1998). The levels of cyclin B1 protein are reduced in these cells and the activity of the ubiquitin pathway that degrades cyclin B is increased. This is consistent with an observation in *Aspergillus* that one of the APC subunits, *bimE*, inhibits the onset of mitosis (James, et al. 1995; Osmani, et al. 1991; Osmani, et al. 1988; Peters, et al. 1996). It will be interesting to determine whether *morula* encodes a component of the APC in *Drosophila* or a regulator that promotes APC activation in polyploid and polytene cells and in diploid cells at the metaphase/anaphase transition.

The transition from G1 to S phase

The transition between G1 phase and S phase in diploid and polyploid cells is especially relevant to my thesis because at this point cell cycle progress is coordinated with developmental events and signals from other cells. The identification of the molecules that induce S phase in budding yeast and multicellular eukaryotes suggests that both transcriptional and post-transcriptional regulatory mechanisms are needed for G1-S progression. Both types of regulation are discussed in budding yeast, mammalian cells, and Drosophila.

Budding yeast and common themes

In the yeast *Saccharomyces cerevisiae* a single cyclin dependent protein kinase Cdc28 (a homologue of fission yeast cdc2) promotes both S and M phase of the cell cycle. To direct passage through distinct transitions of the cell cycle, Cdc28 associates sequentially with cyclins that allow G1/S progression (Cln1 through 3),

cyclins that are needed during S phase (Clb5 and 6), and those that advance mitosis (Clb1 though 4) (for review see Nasmyth 1996). In the presence of nutrients and having reached a critical size, G1 cells initiate DNA replication, form buds, and duplicate their spindle pole body in preparation for subsequent chromosome segregation and cell division. Also, at this point in late G1 (also known as Start) mating factors can no longer induce a haploid cell to exit from the cell cycle and mate.

Progression though Start requires the Cdc28 kinase and at least one of the three Cln cyclins. The inactivation of Cdc28 in G1 cells or mutants lacking all three Cln cyclins arrest in G1, before Start, as large unbudded cells (Richardson, et al. 1989). All three G1 cyclins are very unstable proteins (Cross 1990; Richardson, et al. 1989; Wittenberg, et al. 1990). Mutations resulting in the increased stability of Cln proteins or ectopic expression of the Clns cause G1 budding yeast cells to initiate Start prematurely and at a smaller size than normal, suggesting that the accumulation of Clns may be rate-limiting for Start (Cross 1988; Hadwiger, et al. 1989; Nash, et al. 1988; Sudbery, et al. 1980). Budding yeast cells deleted for any of the *CLN* genes delay in G1 and go through Start at a larger size than their wild-type counterparts (Cross 1988; Hadwiger, et al. 1989; Nash, et al. 1988).

The *CLN1* and *CLN2* genes require an active Cln-Cdc28 kinase for their expression. The *CLN1* and *CLN2* genes are transcribed during late G1, reaching maximum levels just prior to Start, and then decline rapidly (Tyers, et al. 1993; Wittenberg, et al. 1990). *CLN1* and *CLN2* are part of a larger G1-S transcriptional program controlled by two related heterodimeric transcription factors, Swi4/Swi6 and Mbp1/Swi6 (Koch and Nasmyth 1994). Both transcription factors consist of a common regulatory subunit, Swi6, complexed with a DNA binding subunit (Swi4 or Mbp1). Swi4/Swi6 target genes include *CLN1* and *CLN2*. Mbp1/Swi6 is needed for the expression of *CLB5* and *CLB6* (unstable S phase cyclins) as well as numerous other S phase genes (Koch and Nasmyth 1994). Mutants lacking both transcription

factors arrest in the cell cycle in late G1, indicating that passage the G1-S transcriptional program is essential for Start (Koch, et al. 1993). Cln3 (already present in early G1) and Cdc28 jointly trigger the expression of Swi4/Swi6 and Mbp1/Swi6 dependent genes including *CLN1* and *CLN2* (Tyers, et al. 1993). This leads to the synthesis of Cln1 and Cln2 proteins and suggests the possibility of a positive feedback loop for Cln1 and Cln2 production; ectopic expression of any one of the three Clns in a triple *cln1cln2cln3* mutant triggers both Start and the accumulation of *CLN1* and *CLN2* transcripts (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). The abrupt rise in Cln-Cdc28 kinase activity in late G1 (due to positive feedback) is thought to result in an irreversible commitment to enter the cell cycle.

Recent experiments indicate that the role of Cln3 in the regulation of Start differs from that of Cln1 and Cln2. In a *cln3* mutant, G1 cells are unable to activate Swi4/Swi6 and Mbp1/Swi6 target genes until they reach a very large size (Dirick, et al. 1995). In contrast, in a *cln1cln2* double mutant the late G1 transcriptional program occurs in a timely fashion. However, other Start events such as the onset of DNA replication and budding are similarly delayed in both types of mutants. It appears, therefore, that the normal activation of the G1-S transcriptional program requires only the Cln3-Cdc28 kinase (Dirick, et al. 1995). Cln1- and Cln2-Cdc28 kinases are likely to negatively regulate Sic1, an inhibitor of G1-S progression (Dirick, et al. 1995). Sic1 accumulates early in G1, and it is destroyed by ubiquitin mediated degradation shortly before the onset of S phase (Schwob, et al. 1994). The association of Sic1 with S phase cyclin dependent kinases (Clb5 -Cdc28 and Clb6-Cdc28) blocks DNA replication (Mendenhall 1993). Phosphorylation of Sic1 by Cln1- and Cln2-Cdc28 kinases may trigger its ubiquitin dependent proteolysis, permitting entry into S phase (reviewed in King, et al. 1996).

In summary, Cln3 functions as a growth sensor and activates an essential G1-S transcriptional program once cells have reached an appropriate size. In turn, the induced Cln1 and Cln2 proteins turn on the events associated with Start including the degradation of a potent S phase inhibitor.

Mammalian G1/S regulators

Progression from G1 into S phase in mammalian cells is associated with a positive feedback loop controlled both at the post-transcriptional and transcriptional level (reviewed by Sherr 1994; Sherr 1996). In analogy to budding yeast, G1 cyclins trigger a transcription factor (E2F) that in turn activates the rapid synthesis of G1 cyclins and other essential S phase genes. The initial events require growth stimulatory signals, but the established feedback loop is mitogen independent. Furthermore, there are numerous inhibitory proteins that directly target the activity of G1 cyclins and their kinase partners.

The mammalian cyclins needed for S phase include the D-type cyclins (D1, D2, and D3), cyclin E, and cyclin A. These cyclins and their associated cyclin-dependent kinase (cdks) partners may constitute the S phase promoting activity defined in the mammalian cell fusions between G1 and S phase cells. Indeed, ectopic expression of cyclins D1, E, or A shortens G1 and leads to premature entry into S phase (Ohtsubo and Roberts 1993; Quelle, et al. 1993; Resnitzky, et al. 1994; Resnitzky, et al. 1995); however, their functions appear to be distinct. The three D-type cyclins are rapidly induced upon exposure to mitogens, and their assembly with kinase partners cdk4 or cdk6 requires mitogenic signaling (Sherr 1994). Microinjection of antibodies to cyclin D1 prevent entry into S phase when introduced into cultured cells in middle to late G1; injection of cyclin D1 antibodies into cells near to the G1-S boundary, however, does not block S phase, suggesting that the D-dependent kinases execute their S phase promoting function in G1 (Baldin, et al. 1993; Quelle, et al. 1993). In contrast, cyclin

E and cyclin A expression normally peaks at the G1-S boundary of the cell cycle and may be dependent on the E2F transcription factor (Botz, et al. 1996; Geng, et al. 1996; Ohtani, et al. 1995; Shultze, et al. 1995). Both cyclin E and cyclin A activate cdk2, and the inhibition of cyclin E, cyclin A, or cdk2 function prevents entry into S phase (Girard, et al. 1991; Ohtsubo, et al. 1995; Pagano, et al. 1993; Tsai, et al. 1993). It is thought that cyclin D-dependent kinases sense the extracellular environment and trigger G1 progression; cyclin E-cdk2 is downstream of the events initiated by the D-dependent kinases, and cyclin A activates cdk2 shortly after cyclin E at the onset of S phase. In analogy to budding yeast, cyclins D and E perform Cln3-like and Cln1,2 -like functions respectively, and the role of cyclin A is similar to that of Clb5,6.

The activity of D-dependent kinases is likely to promote S phase by inhibiting the function of the retinoblastoma tumor suppressor gene product, RB. The D cyclins bind directly to RB and phosphorylate RB *in vitro* (Ewen, et al. 1993; Kato, et al. 1993). Underphosphorylated RB inhibits exit from G1 and phosphorylation of RB reverses the G1 arrest ability. Notably, in cells that lack functional RB, cyclin D-dependent kinases are dispensable for entry into S phase (Lukas, et al. 1995a) Moreover, ectopic expression of $p16^{INK4a}$ (a specific inhibitor of cdk4/6) causes a G1 arrest in RB-positive but not RB-negative cells (Guan, et al. 1994; Koh, et al. 1995; Lukas, et al. 1995b; Medema, et al. 1995). These findings suggest that RB is the principal substrate of cyclin D-cdk4, cdk6.

The "cyclin D-RB" pathway is likely to be of physiological significance. Inactivating mutations of the RB gene exist in a variety of human tumors. Many tumor cells overexpress cyclin D1, leading to the phosphorylation and functional inactivation of RB. Notably, only one component of the "cyclin D-RB" pathway is altered in tumor cells, suggesting a functional interdependence (Sherr 1996). In addition, transgenic mice lacking cyclin D1 function have phenotypes consistent with cyclin D1 being a positive regulator of G1 progression (Fantl, et al. 1995; Sicinski, et

al. 1995). In *D1 -/-* mice, retinal cell proliferation is reduced and the breast epithelium fails to undergo the massive steroid-induced proliferation associated with pregnancy. The retinal phenotype is striking in that inactivating mutations in the human RB gene cause retinoblastoma.

Several lines of evidence indicate that cyclin E-cdk2 also participates in RB phosphorylation. Ectopic cyclin E expression in human cells causes RB phosphorylation (Hinds, et al. 1992). In addition, cell cycle-dependent phosphorylation of RB is reproduced when RB is ectopically expressed in budding yeast cells. Phosphorylation of RB requires Cln3 and either Cln1 or Cln2; mammalian cyclin D1 can completely complement loss of Cln3, and cyclin E can replace Cln1/Cln2 (Hatakeyama, et al. 1994). This suggests that the D-type cyclins and cyclin E may collaborate in RB phosphorylation. However, it is likely that cyclin E-cdk2 has other critical substrates. In contrast to the D-type cyclins, cyclin E is essential for the G1-S transition in RB-positive and RB-negative cells (Ohtsubo, et al. 1995). In addition, it has been shown that ectopic expression of cyclin E, but not cyclin D1, can override a G1 arrest imposed either by $p16^{INK4a}$ (cdk4/cdk6 inhibitor) or by an RB mutant lacking conserved cdk phosphorylation sites (Lukas, et al. 1997). Thus, cyclin E is involved in S phase promoting events that are downstream of RB phosphorylation.

The E2F transcription factor, a heterodimer of E2F and DP proteins, is a major target of RB action. Hypophosphorylated RB associates with and negatively regulates the activity of E2F (Weinberg 1995). E2F-binding sites are found in the promoters of genes that are required for DNA synthesis and those that regulate S phase entry (Nevins 1992). E2F tethers RB to E2F-dependent promoters during G1, and RB actively represses transcription at these promoters, suggesting that RB-E2F complexes may suppress G1 progression. RB phosphorylation in middle to late G1 releases E2F-binding sites from RB-mediated transcriptional repression, and may

also allow E2F to act as a transcriptional activator at these sites (Qin, et al. 1995; Weintraub, et al. 1995; Weintraub, et al. 1992). Initial RB phosphorylation is likely to be triggered by cyclin D associated kinases in response to mitogenic signals (due to both the timing of cyclin D-cdk function and its strict dependence on RB). During the G1-S transition cyclin E and cyclin A, also rate-limiting for S phase entry, and are likely to take over phosphorylation of RB (for review see Bartek, et al. 1996). Because the genes encoding cyclin E and E2F-1 itself are E2F-dependent, a positive feedback loop may ensue (Botz, et al. 1996; Geng, et al. 1996; Johnson, et al. 1994; Ohtani, et al. 1995). The rapid increase in liberated E2F is thought to result in an irrevocable commitment to enter S phase and to proliferate (Sherr 1996).

Numerous studies in mammalian cell lines implicate E2F as a critical regulator of G1-S progression. However, it is not clear whether the predominant role of E2F *in vivo* is to advance cells into S phase or suppress G1 progression. Ectopic expression of the best characterized E2F protein, E2F-1, is sufficient to drive serum starved, quiescent fibroblasts through G1 and into S phase, suggesting that E2F is a potent activator of G1-S progression (Johnson, et al. 1993; Qin, et al. 1994). In addition, it was shown that the induction of DNA synthesis by E2F-1 is dependent on its transcriptional activation domain (Hsieh, et al. 1997; Phillips, et al. 1997; Qin, et al. 1995). Furthermore, overexpression of dominant negative DP-1 mutant results in a G1 arrest (Wu, et al. 1996). These findings indicate that E2F-1 may be a transcriptional activator of G1-S progression in normal cells.

Mice lacking functional E2F-1 exhibit phenotypes that are opposite of those predicted by the overexpression studies. Instead of defects indicative of a failure to proliferate, the E2F-1 knockout mice develop a variety of tumors, suggesting a tumor suppressor function (Field, et al. 1996; Yamasaki, et al. 1996). E2F-1 appears either not to be critical for entry into S phase or other E2F proteins (E2F-2 through -5) can compensate for this function. The primary role of E2F-1, therefore, may be to tether

RB to E2F-dependent promoters and to repress their utilization during G1. Recent studies suggest that RB can affect the state of chromatin condensation though an interaction with a histone deacetylase, and already bound E2Fs may serve as docking sites (Brehm, et al. 1998; Luo, et al. 1998; Magnaghi-Jaulin, et al. 1998).

Once cells enter S phase, cyclin A-cdk2 phosphorylates DP-1, thereby precluding E2F from binding to DNA. The timely dissociation of E2F from E2F-dependent promoters appears to be important for S phase progression, since inappropriate binding of E2F to DNA (due to a failure to interact with cyclin A) causes S phase arrest (Krek, et al. 1995). The mechanism by which E2F blocks S phase is not understood, and it is not clear that this ability is utilized by normal cells to control DNA replication.

S phase regulation in Drosophila embryos

Drosophila homologues of key mammalian G1-S regulators have been identified, suggesting that the regulatory mechanisms may be conserved. These homologues include G1 cyclins D and E, cdks 2 and 4, cyclin-dependent kinase inhibitor p21 (*dacapo*), cyclin A, an RB-like protein (RBF), and both the E2F and DP subunits of the E2F transcription factor (de Nooij, et al. 1996; Du, et al. 1996; Dynlacht, et al. 1994; Finley, et al. 1996; Hao, et al. 1995; Knoblich, et al. 1994; Lane, et al. 1996; Lehner and O'Farrell 1989; Ohtani and Nevins 1994; Richardson, et al. 1993; Sauer, et al. 1995; Sauer, et al. 1996). Mutations in some of these genes have been reported and provide important insights into their *in vivo* functions during the course of development.

Transcriptional regulation of S phase appears not to be important in early embryos. Drosophila development begins with 13 extremely rapid, synchronous S-M cycles that lack both gap phases. These nuclear divisions occur in a syncytium and depend on maternally supplied mRNAs and proteins. The rapidity of the cycles

precludes zygotic transcription. During cycle 14 cellularization takes place, a G2 phase is added to the cell cycle, and zygotic gene expression occurs. Mitotic divisions are no longer synchronous and groups of cells that differentiate into the same tissue undergo mitosis as a domain (Foe 1989). These divisions are preceded by developmentally-induced transcription of the Drosophila *string* gene *(cdc25)*, a rate limiting activator of *cdc2* (Edgar, et al. 1994; Edgar and O'Farrell 1990). In contrast, the expression of replication functions is not developmentally regulated, and S phase immediately follows mitosis, suggesting that post-transcriptional mechanisms continue to control the initiation of S phase.

After mitosis16, a G1 phase is observed for the first time in Drosophila embryogenesis. The addition of G1 correlates with the onset of a major cell cycle divergence. The cells in the epidermis exit from the cell cycle and arrest in G1. The embryonic precursors to the polytene larval tissues also arrest in G1 and then, initiate the endo cell cycle in invariable tissue-specific domains (Smith and Orr-Weaver 1991). In contrast, the embryonic neural cells continue to undergo mitotic divisions. These developmental changes are reflected in the transcriptional regulation of *cyclin E* and genes encoding replication functions: proliferating cell nuclear antigen (*PCNA*), ribonucleotide reductase 1 (*RNR1*), *RNR2*, and *pola* (Duronio and O'Farrell 1994). During cycles 14-16, these genes are expressed at constitutively high levels. After mitosis 16, expression is down-regulated in all tissues with the exception of the nervous system, and transcripts accumulate prior to the onset of S phase in the endo cycle. The spatio-temporal pattern of endo cycle S phase and the anticipatory transcription of S phase genes are controlled by developmental inputs rather than cell cycle progress (Duronio and O'Farrell 1994; Smith and Orr-Weaver 1991). In *string* mutant embryos, the dynamic expression pattern of S phase genes (including cyclin E) and the subsequent endo cycle S phases occur normally, even though the preceding mitotic cycles (14-16) do not occur.

Mutations in the *cyclin E* gene block S phase after mitosis 16, and ectopic *cyclin E* expression is sufficient to trigger S phase *in vivo* (Duronio and O'Farrell 1994; Knoblich, et al. 1994). It appears, therefore, that cyclin E is both needed and rate-liming for DNA replication in mitotic and endo cycle cells. It is likely that *cyclin E* is needed prior to cycle 16 as well, but that maternal stockpiles of *cyclin E* transcript and/or protein suffice (Richardson, et al. 1993). Mutations in the gene encoding the catalytic partner of *cyclin E*, *cdk2* (encoded by the *cdc2c* gene), have not been reported. However, Drosophila cdk2 and cyclin E form an active kinase complex (Knoblich, et al. 1994; Sauer, et al. 1995). Thus, the block to S phase in the *cyclin E* mutant is likely to result from loss of cdk2/cyclin E function.

In analogy to mammalian cells, Drosophila p21 (encoded by *dacapo*) has been shown to inhibit the *in vitro* kinase activity of human and Drosophila cyclin E-cdk2 complexes (de Nooij, et al. 1996; Lane, et al. 1996). Indeed, strong mutations in *dacapo* or ectopic *cyclin E* expression cause the normally G1 arrested epidermal cells to undergo an inappropriate round of S phase. Thus, it appears that the down-regulation of *cyclin E* levels after the first 16 cycles and the inhibition of residual cyclin E-cdk2 activity by p21 contribute to G1 arrest. This is also consistent with the induction of *dacapo* transcripts and protein in epidermal cells as they exit from the cell cycle.

The identification of Drosophila E2F and RBF (an RB-related protein) as well as their biochemical properties provide further evidence of the similarity between the G1-S pathway in flies and mammals. RBF has been shown to associate with E2F/DP, and it inhibits E2F/DP dependent transcriptional activation (Du, et al. 1996). In addition, RBF can be phosphorylated by the cyclin E-cdk2 kinase *in vitro* (Du, et al. 1996). The *in vivo* function of RBF remains unclear as the phenotypic consequences of disrupting RBF function have not been reported. However, ectopic expression of *dE2F* and *dDP* in embryos results in the induction of S phase genes and

causes normally quiescent cells to enter S phase (Duronio, et al. 1996). In wild-type embryos *dE2F* is required for the G1-S expression of *cyclin E*, *PCNA*, and *RNR2*; these transcripts are missing in the mitotic and endo cycle tissues of *dE2F* mutants (Duronio and O'Farrell 1995; Duronio, et al. 1995). This effect is likely to be direct, since E2F binding sites have been found in the promoters of Drosophila *PCNA* and *pola* genes (Ohtani and Nevins 1994; Yamaguchi, et al. 1995). My study reports mutations in *dDP* and *dE2F* (including a mutation lacking the conserved C-terminal transcriptional activation and RB-binding domain of *dE2F*) and explores the impact of *dE2F* dependent transcription on S phase entry and development.

S phase control during Drosophila oogenesis

The mechanisms controlling S phase entry in mitotic and polyploid cells are being rapidly revealed. In contrast, very little has been learned about the regulation of origin activity during S phase. In a variety of systems, cytological and molecular analyses suggest that not all origins are active within a particular round of S phase. In addition, it is clear that different origins initiate replication at different times in S phase. The timing of origin activation appears to be affected by chromosomal context and gene expression. These observations suggest that developmental inputs may determine the timing and choice of replication origins (reviewed by Fangman and Brewer 1992).

Studies in Drosophila reveal that the regulation of origin activity during S phase becomes increasingly complex as development proceeds and cells differentiate (see Spradling and Orr-Weaver 1987). In early Drosophila embryos the entire S-M cycle is completed in 8-10 minutes, with S phase being approximately 3.4 minutes long. To duplicate the entire genome in 3.4 minutes, origins are closely spaced and activated simultaneously at the start of S phase (Blumenthal, et al. 1973). Similar results were reported from studies of DNA replication in other dipterans (Lee and

Pavan 1974; Zakian 1976). At the onset of cellularization and full-blown zygotic transcription in Drosophila embryos, the cell cycle and S phase are greatly lengthened with S phase being 20 minutes long during cycle 14 (McKnight and Miller 1976; McKnight and Miller 1977). The longer S phase is likely to result from the appearance of asynchrony in the activation of origins. The switch to temporal regulation of origin activity is attributed to global changes in chromosome structure at the time of zygotic transcription. In addition, the number of origins utilized during S phase may decrease when heterochromatin is formed. Studies of DNA replication in Xenopus embryos reveal that the zone where replication is initiated becomes restricted during the mid-blastula transition, the onset of zygotic transcription (Hyrien, et al. 1995).

In Drosophila larval brain cells (diploid mitotic tissue) there is a dramatic reduction in the number of origins used, and it takes approximately 10-12 hours to duplicate the genome (Spradling and Orr-Weaver 1987). The elongation rates, however, are similar to those observed in embryos. Moreover, heterochromatic regions replicate late in S phase. Studies of DNA replication in larval polytene cells also reveal that replication occurs in a discrete temporal and spatial pattern: euchromatic sequences are replicated in early to middle S phase, and heterochromatic sequences replicate in middle to late S phase. In addition, polytene cells underrepresent 20-30% of DNA due to incomplete replication of heterochromatin (Hammond and Laird 1985a; Lilly and Spradling 1996; Rudkin 1969). Early and late replicating domains are also observed in yeast and mammalian cells (McCarroll and Fangman 1988; O'Keefe, et al. 1992), suggesting that the temporal and spatial control of origin activity is a conserved feature of eukaryotic replication.

The Drosophila ovary is a useful tissue to identify the genes involved in differential replication control. There are only a few cell types and their behavior is

well characterized. Phenotypic analysis is facilitated both by the depletion of maternal pools of most cell cycle regulators and components of the DNA replication machinery in the adult as well as assays to monitor the parameters of replication *in situ*. This is in contrast to the embryo and larva where maternally supplied wild-type messages and products persist, making it difficult to define mutant phenotypes precisely. In addition to the depletion of maternal products in the adult, the intensive preparatory events for embryogenesis that occur in the ovary (polyploidization and amplification of chorion gene clusters) place great demands on replication factors. Weak alleles of essential functions for replication cause cell cycle defects in oogenesis, resulting in sterility. Hypomorphic mutations in *cyclin E*, *PCNA*, and *orc2* are all female sterile (Henderson, et al. 1994; Landis, et al. 1997; Lilly and Spradling 1996).

Oogenesis begins with four mitotic divisions that produce a 16 cell egg chamber cyst. The process of cytokinesis is incomplete, so the 16 cells are interconnected by cytoplasmic bridges known as ring canals. These cells subsequently enter a long S phase (24 hours or more) (Spradling 1993). This constitutes the premeiotic S phase of the future oocyte, and as in other organisms, this replication event requires the most time. Autoradiography studies suggest that premeiotic S phase is also temporally regulated: euchromatin replicates first, then replication of euchromatin and heterochromatin overlap, followed by labeling only within heterochromatin (Carpenter 1981). Only one of the 16 cells continues through meiosis and becomes the oocyte; the other 15 cells (nurse cells) enter the endo cell cycle from G2 and grow via polyploidization (Spradling 1993). The relationship between oocyte determination and the cell cycle is poorly understood. However, a mutation in *cyclin E* causes a nurse cell to become a second oocyte (Lilly and Spradling 1996). Thus, differential distribution of cell cycle regulators may play a role in oocyte determination.

During polyploidization the nurse cell genome is replicated differentially and heterochromatic sequences are underrepresented (see Spradling and Orr-Weaver

1987). Staining with the DNA dye DAPI reveals one or a few small, intensely labeled chromocenters (aggregates of heterochromatic sequences) in each nurse cell nucleus. In a female sterile *cyclin E* mutant the size of the chromocenter is greatly increased due to restored late replication (Lilly and Spradling 1996). The increased presence of nurse cell heterochromatin due to partial loss of cyclin E function suggests that *cyclin E* differentially regulates the activity of DNA replication origins in polyploid cells (in addition to its general requirement for entry into S phase). It is not clear if *cyclin E* is involved in temporal control of origins in diploid cells.

It has been proposed that the extent of underrepresentation in polyploid nurse cells is determined by the length of S phase in these cells. The relative over-replication of heterochromatic sequences in the *cyclin E* mutant as compared to wild-type nurse cells may result from a longer S phase in the mutant (Lilly and Spradling 1996) If robust oscillations of cyclin E are needed to stimulate the downregulation of S phase, S phase may be prolonged due to dampened oscillations of cyclin E protein during the nurse cell endo cell cycles in the mutant. During the lengthened S phase, sequences that replicate late and are not normally replicated in wild-type nurse cells could be replicated. There is no evidence that S phase is actually prolonged in the mutant. However, if this mechanism turns out to be correct, the role of cyclin E in the control of differential replication would be rather indirect. A recent study shows that cyclin E accumulates at amplification sites in follicle cell nuclei, suggesting that cyclin E may play a direct role in the activation and/or repression of origins (Calvi, et al. 1998). The identification of other molecules affecting nurse cell endo cycles will help clarify the molecular mechanism underlying differential replication.

Differential replication has also been detected in ovarian follicle cells. Follicle cells surround the developing egg chamber cyst and undergo several mitotic divisions to generate a monolayer of approximately 1000 cells before becoming polyploid (Spradling 1993). The follicle cells carry out 4-5 endo cycles and underrepresent late-

replicating DNAs. The extent of underrepresentation is more severe in polyploid follicle cells than nurse cells, suggesting that developmental inputs modulate this process (Hammond and Laird 1985a). The difference between follicle cell and nurse cell underrepresentation is largely due to the more complete replication of late-replicating rDNA genes in nurse cells. This is suggestive, since nurse cell synthesize all the rRNA of the future embryo within oogenesis.

The relationship between replication and tissue-specific function is best exemplified in Drosophila by the amplification of chorion gene clusters in follicle cell nuclei. Amplification is required for the rapid synthesis of the eggshell (chorion) that protects the embryo during its first 24 hours of development. The chorion genes are only transcribed in the follicle cells and only after multiple copies of these genes have been generated (reviewed by Orr-Weaver 1991). Mutants shown to be defective in amplification (by quantitative Southern blots of egg chamber DNA) cause females to be sterile due to synthesis of thin and inadequate eggshells for their eggs (Spradling 1993).

Amplification of chorion genes has been shown to occur in two phases (Calvi, et al. 1998). Amplification of at least one of the chorion clusters begins during the last follicle cell endo cycles and appears to be restricted to S phase of the endo cycle. Most amplification, however, occurs after the cessation of genomic replication. The transition from general amplification to amplification of chorion genes is readily observed by BrdU labeling of whole-mount ovaries. During genomic replication, BrdU is incorporated throughout the entire follicle cell nucleus, and groups of follicle cells in the same egg chamber undergo BrdU incorporation at different times to give an asynchronous pattern: both labeled (S phase) and unlabeled (G phase) follicle cell nuclei are observed. The cessation of genomic BrdU incorporation is followed by continuous amplification of chorion genes: BrdU is incorporated in all the follicle cells at the same time but only at four subnuclear foci. The subnuclear BrdU foci

correspond to amplifying regions of the genome (Calvi, et al. 1998). The continuous incorporation of BrdU at amplifying chorion clusters is consistent with several lines of evidence showing that these genomic interval escape restriction on replication, including the detection of clustered replication forks in EM spreads (Osheim, et al. 1988).

Summary

The transition from G1 to S phase in dividing and polyploid cells is a critical cell cycle control point. Developmental inputs converge on S phase activators that phosphorylate key substrates. In addition, many essential S phase genes appear to be transcriptionally regulated and accumulate at the G1-S transition. In polyploid cells, DNA replication occurs in the absence of mitosis. Thus, in addition to the G1-S activities, polyploid cells must permanently shut off all aspects of mitosis. Polyploid cells also alter the parameters of S phase such that differential replication is readily observed. These cells allow us to evaluate the roles of S phase players in the temporal and spatial control of replication origins within S phase.

This thesis examines the control of DNA replication in a number of different developmental contexts. Chapter 2 presents mutations in new and known genes that affect a G1-S transcriptional program in mitotic and/or endo cycle cells in late Drosophila embryogenesis. In Chapter 3, mutations in *dDP* are presented and used to examine the relationship between E2F-dependent G1-S transcription and S phase onset *in vivo*; the role of *dDP* and *dE2F* in cell cycle progression in mitotic and polytene tissues is described both in embryos and at later developmental stages. Finally, Chapter 4 presents studies in polyploid cells of the adult ovary that reveal a requirement for E2F/DP in the differential control of DNA replication origins and developmentally-induced apoptosis.

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Chapter Two

Screen for mutations that disrupt a G1-S transcriptional program in late embryogenesis

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*IR worked out the method for *in situ* hybridization on large numbers of independent lines, established and screened half of the second chromosome lines, screened all of the third chromosome lines, carried out the BrdU studies and the analysis of the mitotic mutants.

Introduction

The first detectable G1 period in Drosophila development occurs after mitosis 16 in the embryo. It is coincident with the onset of a cell cycle divergence in three types of tissues: the epidermis exits the cell cycle, the neural and imaginal cells continue to undergo mitotic divisions, and the cells giving rise to most of the larval tissues initiate polytenization. Polyteny results from the endo cycle, a modified cell cycle in which an S phase alternates with a gap phase in the absence of mitosis (Smith and Orr-Weaver 1991). Both the onset and the parameters of the endo cycle are regulated developmentally (Smith and Orr-Weaver 1991). The length of the G1 phase after mitosis 16 and prior to the first polytene S phase differs considerably between tissues, resulting in a dynamic spatio-temporal pattern of DNA synthesis. The duration of endo cycle gap phases and the final levels of polytenization also vary from tissue to tissue in a stereotypic manner.

It has long been thought that the switch from a mitotic cell cycle to polytenization requires at least one global regulator controlling the onset of endoreplication in all polytene tissues. In addition, multiple tissue-specific regulators of endoreplication may exist. To date, however, mutations in genes specific for the onset of polytene replication have not been isolated. 34% of the Drosophila genome has been screened for loci that affect DNA replication late in embryogenesis using overlapping deficiency stocks (Smith, et al. 1993). This systematic search for S phase regulators yielded no endo cycle specific functions, but three intervals affecting replication in polytene and diploid tissues were found. One of the intervals correlates with the deletion of the histone genes. It is not clear whether the other two intervals encode regulatory functions for replication or components of the replication machinery.

To date, *cyclin E* is the only known regulator of the G1-S transition that is limiting for S phase in late embryogenesis (Knoblich, et al. 1994; Richardson, et al.

1993). Mutations in the *cyclin E* gene block S phase after mitosis 16 in mitotic and endo cycle cells (Knoblich, et al. 1994). The requirement for *cyclin E* function correlates with the onset of a G1-S transcriptional program that includes *cyclin E* and several other S phase genes: *proliferating cell nuclear antigen* (*PCNA*), *DNA polymerase α*, and the two *ribonucleotide reductase* subunits (*RNR1* and *RNR2*) (Duronio and O'Farrell 1994). These S phase functions are expressed constitutively prior to mitosis 16. However, upon addition of a G1 period, expression of these genes is restricted to late G1 and S phases of the cell cycle and parallels the spatio-temporal pattern of S phase in late Drosophila embryogenesis (Duronio and O'Farrell 1994). In analogy to other systems, we reasoned that the induction of essential S phase genes at the G1-S boundary reflects a decision to progress through the cell cycle. The transcriptional pattern of these S phase reporter genes, therefore, could be used to identify genes that regulate S phase entry in mitotic and endo cycle cells.

We devised a method for doing *in situ* hybridizations on large numbers of independent mutagenized lines and screened for zygotic mutations that alter the expression of endogenous *PCNA* transcripts in polytene and/or mitotic tissues in late embryogenesis. The ability to examine S phase in mitotic and polytene tissues during the same developmental period made it possible to distinguish between regulators specific to the endo cell cycle and a set of G1/S genes that are common to the mitotic and endo cell cycles. *PCNA* was chosen as the S phase reporter in the primary screen because it is expressed well in both mitotic and polytene tissues. The levels of *RNR2* transcripts are low in the CNS, and *cyclin E* transcripts are present at low levels in the endodomains, making it difficult to examine S phase in mitotic and polytene cells simultaneously. However, both *RNR2* and *cyclin E* were used in secondary screens in order to distinguish mutations specific to *PCNA* expression from those that affect the coordinate expression of S phase genes at the G1-S boundary. Mutations in genes that affect only *PCNA* transcription should show normal spatio-

temporal expression of the other S phase reporters. In addition, it is important to note that alterations in *PCNA* transcription would be detected only in embryos mutated in genes for which maternal gene products do not persist late into embryogenesis.

We expected to identify a variety of zygotic mutants in which the expression of *PCNA* is affected. The mutant embryos would fall into three morphological classes: (1) mutants with gross developmental abnormalities; (2) mutants with morphological abnormalities suggestive of a failure to complete the postblastoderm mitotic divisions (cycles 14-16); and (3) mutants that are morphologically normal. The majority of the mutants were expected to fall into the first class, but their effect on *PCNA* expression is likely to be indirect. The second class was expected to define essential mitotic functions. Seven genes are known from previous work to encode products necessary for progression through cycles 14-16: *string*, *pimples*, *pebble*, *three rows*, *fizzy*, *cyclin A* and *l(2)51Ec* (for review see Orr-Weaver 1994). We expected to recover additional alleles of these genes as well as new genes. The third class of mutants were of greatest interest, since mutations that block the cell cycle after mitosis 16 are expected to be morphologically normal, an expectation that derives from the normal appearance of *cyclin E* mutant embryos (Knoblich, et al. 1994). This class of mutants should include mutations that cause a block to replication in mitotic and polytene tissues or polytene tissues specifically.

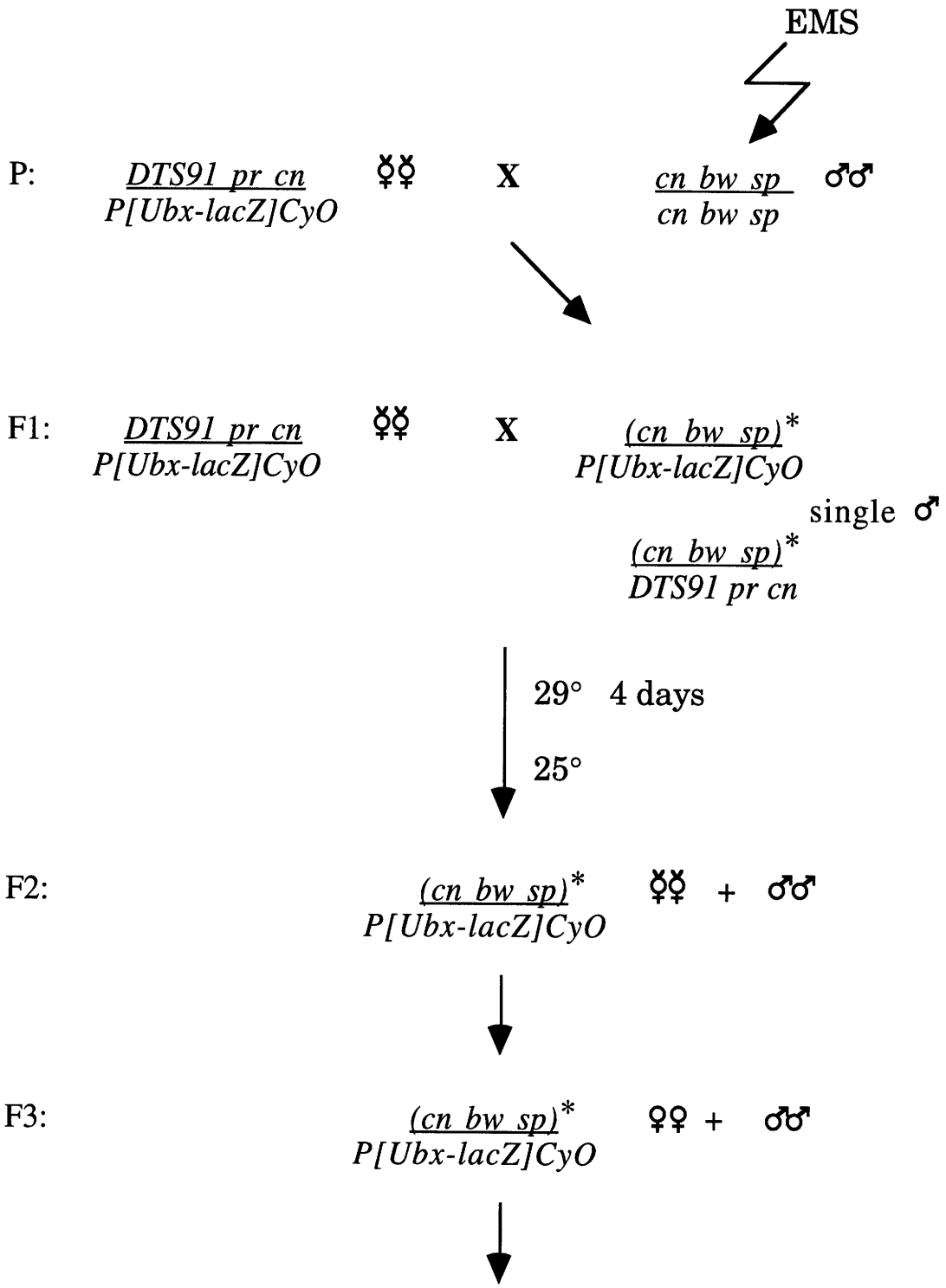
Results

Genetic screen

We mutagenized flies with EMS and established 3010 second chromosome lines, each representing a single mutagenized chromosome with an average of 2.7 lethal hits (Fig. 1). In addition, 1000 third chromosome lines were provided by the Lehmann lab (Moore, et al. 1998). The mutagenized chromosomes were maintained over a balancer chromosome marked with a *Ubx-lacZ* transgene to distinguish between embryos homozygous for the mutagenized chromosome and their heterozygous siblings. Embryos were collected from independent mutant lines, staged appropriately, and hybridized *in situ* with labeled *lacZ* and *PCNA* riboprobes simultaneously (embryos that did not express *lacZ* were homozygous for the mutagenized chromosome). Hybridizations were performed in mass in multi-well dishes. Then, embryos from each line were mounted and examined microscopically for alterations in endogenous *PCNA* expression in polytene and mitotic tissues (Fig. 2B). The embryos that were heterozygous for the mutagenized chromosomes expressed *lacZ,* serving as an internal control for the efficacy of the staining reaction (Fig. 2C). In addition, the pattern of *lacZ* expression did not obscure the spatio-temporal pattern of *PCNA* transcripts. This made it possible to ascertain whether the mutations were recessive or dominant.

The results from the screen of the second and third chromosome are summarized in Table 1. The mutants were categorized into three classes: gross morphological defects, postblastoderm defects, and morphologically normal. The mutants with gross morphological defects were not maintained because their affect on the pattern of *PCNA* transcription is likely to be indirect. The other two classes of mutants were kept for further study. The majority of the mutants that appeared to arrest during the mitotic postblastoderm divisions (cycles 14-16) did not affect the

Figure 1. Screen for mutations on the second chromosome that disrupt G1-S transcription in mitotic and/or polytene cells. The crosses to establish independent mutagenized lines are shown. Isogenized *cn bw sp* males were mutagenized with EMS and the mutagenized chromosomes were ultimately balanced over a chromosome marked with a *Ubx-lacZ* transgene. From the F3 adults, 8- to 15-hr embryos were collected and hybridized in situ with *PCNA* and *lacZ* riboprobes. Mutant lines that produced embryos with altered *PCNA* expression or that had morphological defects consistent with an arrest during cycles 14-16 were maintained for further analysis.



Collect embryos and examine by in situ hybridization.

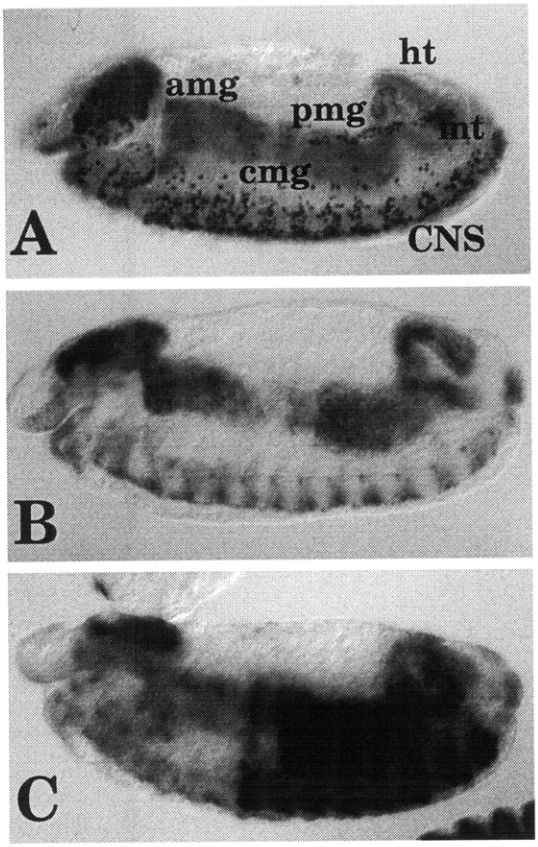


Figure 2. *PCNA* expression was used as the reporter in a genetic screen.

(A) The spatio-temporal pattern of DNA replication is shown in a wild-type 10.5-hr embryo. The darkly staining cells are those that incorporated BrdU during a 40-min pulse. The mitotically proliferating central nervous system (CNS), the endoreplicating anterior midgut (amg), central midgut (cmg), posterior midgut (pmg), hindgut (hg), and malphigian tubules (mt) incorporate BrdU.

(B) The transcription pattern of *PCNA* precisely matches the spatio-temporal pattern of BrdU incorporation. *PCNA* transcripts were detected by whole-mount in situ hybridization. A wild-type 10.5-hr embryo is shown.

(C) A 10.5-hr embryo from a wild-type line maintained over a balancer chromosome marked with a *Ubx-lacZ* transgene is shown. Both *PCNA* and *lacZ* transcripts were detected in heterozygous embryos by whole-mount in situ hybridization. *LacZ* expression is observed in the posterior half of the embryo (right) in the epidermis and does not obscure the expression of *PCNA* in the endodomains.

Table 1: Results from *in situ* hybridization screen

	second chromosome	third chromosome
Lines scored	3010	1000
Lines selected	160	15
Morphological Classes:		
gross abnormalities	137	11
post blastoderm defects	11	1
normal morphology	12	3

expression of *PCNA*. These mutations were maintained because they may provide insight into the regulation of mitosis. However, four mutations with morphology indicative of a failure to progress through the three postblastoderm divisions did affect the expression of *PCNA*. These mutations are likely to be needed for replication during cycles 14-16. In twelve of the mutagenized second chromosome lines and three third chromosome lines, *PCNA* expression was altered but the morphology of the homozygous mutant embryos was normal (Table 1). These mutations are of greatest interest because they control *PCNA* transcription at a developmental stage when most tissues initiate endo cycles.

Morphologically normal mutants

Seven of the second chromosome lines failed to express *PCNA* transcripts in mitotic and polytene tissues at wild-type levels (Fig. 3B,C). These lines had similar effects on *RNR2* expression and showed a reduction in the levels of the *cyclin E* transcripts in the endodomains. The expression of *PCNA* in five of the mutations was almost identical to that previously described for mutations in the *dE2F* gene (Fig. 3B) (Duronio, et al. 1995). Given the similarity between the *dE2F* phenotype and that of our mutants, we tested whether a deficiency known to delete the *dDP* gene also uncovered these five mutants. All five mutations failed to complement the *dDP* deletion. The position and nature of the *dDP* mutations was determined and is shown in Chapter 3. These five mutations provide the first opportunity to examine the *in vivo* role of this gene (see Chapters 3 and 4). The other two mutations constitute a complementation group that appears to be novel.

Five mutations failed to downregulate the expression of *PCNA* in the endodomains (Fig. 3D). *PCNA* transcription is activated normally, but the transcripts persist inappropriately. This phenotype was observed previously for *cyclin E* (Duronio and O'Farrell 1995; Sauer, et al. 1995), and all five mutations failed

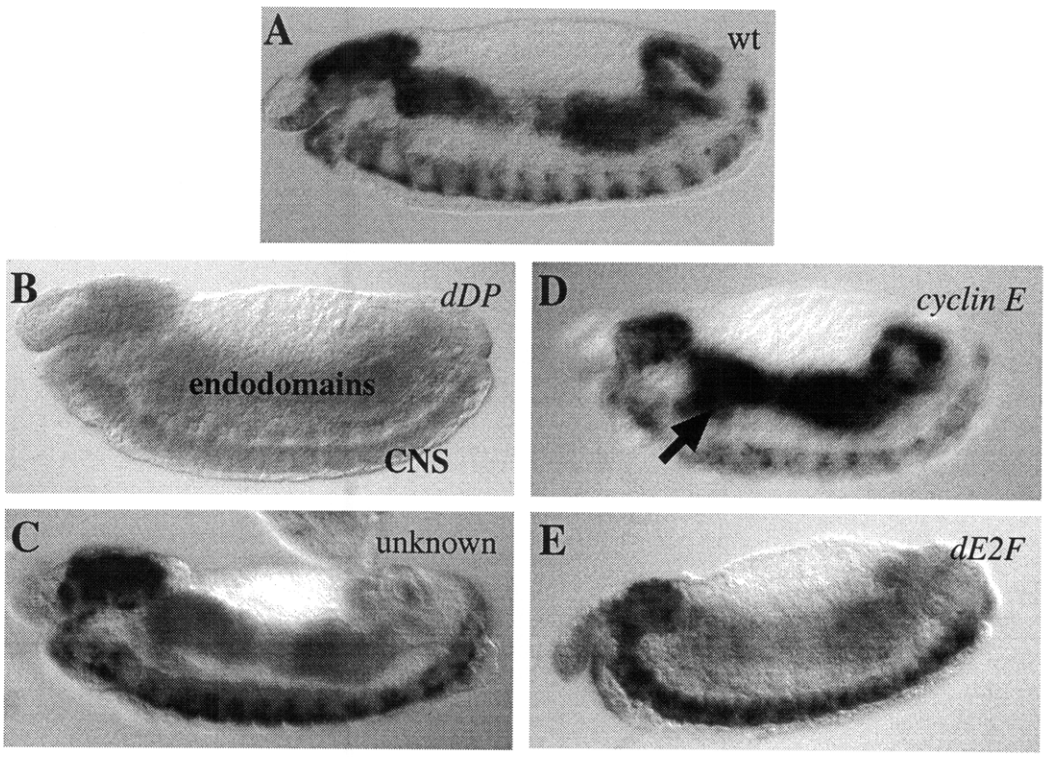


Figure 3. *PCNA* expression in mutant embryos with normal morphology. *PCNA* transcripts were detected by whole-mount in situ hybridization.

(A) *PCNA* transcripts in a wild-type 10.5-hr embryo.

(B) *PCNA* is expressed at greatly reduced levels in the central nervous system (CNS) and the endodomains of *10b* (dDP^{a4}) homozygous mutant embryos. This complementation group is the Drosophila *DP* gene (5 alleles).

(C) *PCNA* transcripts are diminished especially in the endodomains of *3.47* mutant embryos (2 alleles), but not to the degree observed in the *dDP* mutants.

(D) *PCNA* transcripts are not downregulated in the endodomains of *32a* homozygous mutant embryos. The continued presence of *PCNA* transcripts in the salivary gland (arrow) is inappropriate for this developmental point. This mutation and four others fail to complement the *cyclin E* gene. In the allele being shown the cells in the CNS are larger than normal and possibly polyploid.

(E) *PCNA* expression is specifically off in endo cycle cells of *1q19* ($dE2F^{i1}$) mutant embryos. This mutation and one other are new alleles of *dE2F*.

to complement each other and strong mutations in the *cyclin E* gene. Allelism was determined based on the failure to recover transheterozygous viable progeny as well as the failure to downregulate *PCNA* transcription in the endodomains of transheterozygous embryos. All five of the mutations are lethal *in trans* to strong alleles of *cyclin E*. However, one of the mutations (*1f36*) appears to be weaker than the others. In addition, one of the putative *cyclin E* alleles (*32a*) has enlarged cells in the CNS and may provide new insights into the activity of cyclin E, if this phenotype is indeed linked to the putative *cyclin E* mutation. The replication phenotypes of *32a* and the other new alleles could be informative.

From the third chromosome lines, two mutants were recovered with reduced levels of *PCNA* expression in polytene tissues (Fig. 3E). This mutant phenotype was predicted for an endo cycle specific regulator. These two mutations fully complement each other. However, the transheterozygous embryos were not examined by *in situ* hybridization. To our surprise, both mutations affect the *dE2F* gene (see Chapter 4), although *dE2F* is essential in both mitotic and polytene tissues (see Chapter 3). One of the new *dE2F* alleles is fully viable *in trans* to a *dE2F* deficiency. The other mutation is semi-lethal. We also recovered a third chromosome mutant line that affects *PCNA* expression in mitotic and polytene tissues. This may be another allele of *dE2F* or a new gene.

Postblastoderm mutants

In addition to the mutants discussed above, a number of known and novel genes that are required during the mitotic postblastoderm divisions (cycles 14-16) were isolated. These mutants were readily identified due to the characteristic small size of the embryos and neural cells that appear polyploid (Fig. 4 A,B). Seven of the mutated second chromosome lines were tested for lethality *in trans* to *fizzy*, *three rows*, and *pimples*. These complementation tests revealed that two of the lines failed

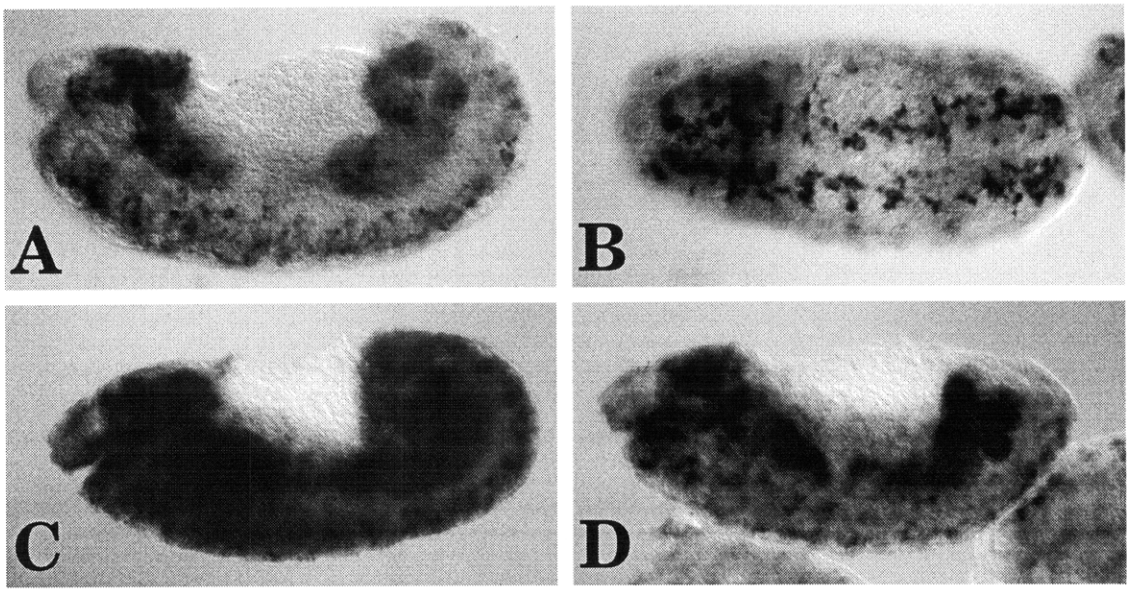


Figure 4. *PCNA* expression in mutants that are arrested during cycles 14-16. *PCNA* transcripts were detected by whole-mount in situ hybridization.

(A) In this 10.5 hr homozygous mutant embryo (*249*), *PCNA* is expressed in the normal pattern but the embryo is smaller and the cells in the central nervous system become polyploid. This mutation fails to complement alleles of *pimples*, a gene that acts in mitosis to promote the separation of sister chromatids (Stratmann and Lehner, 1996).

(B) A ventral view of a homozygous mutant embryo from the line shown in A. The defect in the nervous system is very distinct; this class of mutants were therefore readily identified in this screen.

(C) In this homozygous mutant embryo (*19K*), *PCNA* transcripts persist inappropriately in the epidermis. This mutation is part of the *l(2)51Ec* complementation group and there are three other new alleles.

(D) A 10.5 hr homozygous mutant embryo is shown from the same line (*19K*). The epidermal *PCNA* expression is reduced and the normal spatio-temporal pattern of *PCNA* transcription can be seen in the endodomains.

to complement *pimples* and another line failed to complement *three rows*. Both *pimples* and *three rows* act in mitosis to promote the separation of sister chromatids ((Stratmann and Lehner 1996). The mutants fail to undergo cytokinesis, and as a consequence the neural cell are greatly enlarged. The other four lines complement each other and may define several new functions required during cycles 14-16. From the third chromosome lines, one line appears to be arrested during cycles 14-16. This mutation is likely to define a new gene since there are no third chromosome mutations known to affect the postblastoderm divisions.

We also isolated four second chromosome mutations that arrest during cycles 14-16 but appear to affect replication rather than chromosome segregation. These four mutations fail to complement each other and *l(2)51Ec*. *l(2)51Ec* has already been identified as having a replication phenotype during the postblastoderm divisions (Smith, et al. 1993). The new mutants and *l(2)51Ec* fail to downregulate constitutive transcription of S phase genes in the epidermis (Fig. 4C). Developmentally regulated G1-S transcription in endo cycle tissues was observed in the correct pattern (Fig. 4D). In addition, DNA replication, as gauged by incorporation of BrdU, seems to persist in the normally arrested epidermis after mitosis 16 (compare Fig. 5B to 5A). The BrdU labeling was punctate and did not fill the entire nucleus (compare Fig. 5B' to 5A'). This staining pattern is suggestive of continued late replication of S phase 15 or 16 rather than a new round of S phase. In the endodomains DNA replication was mostly blocked (Fig. 5B).

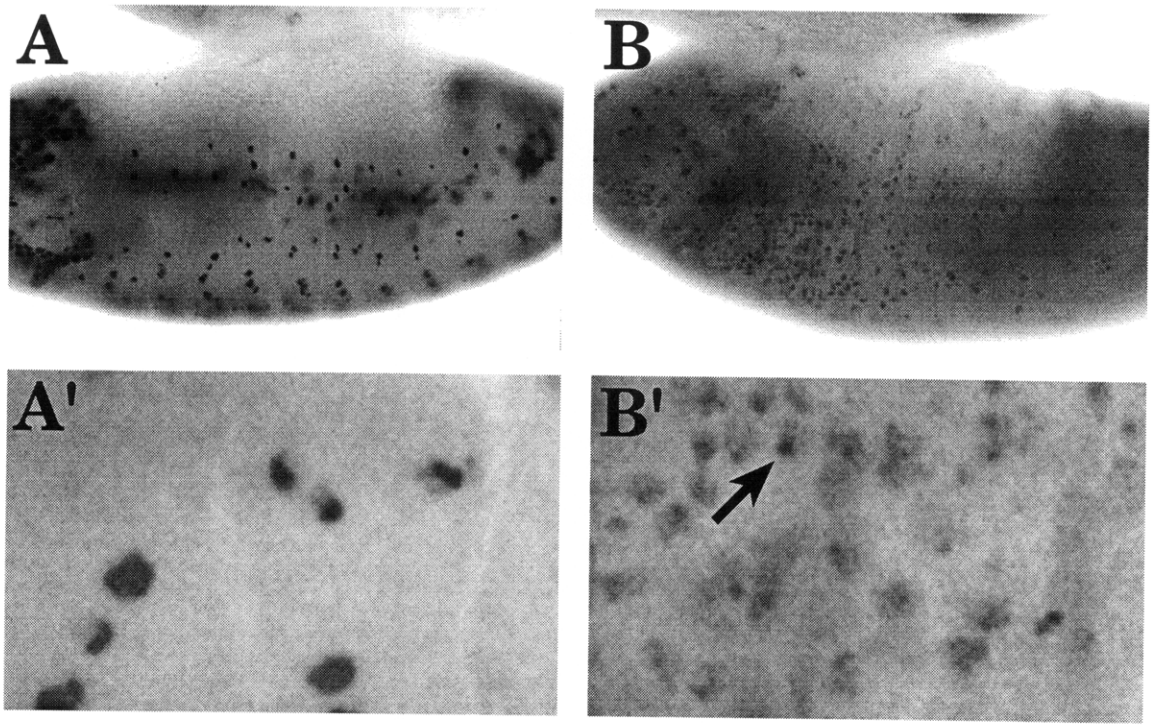


Figure 5. In the *l(2)51Ec* complementation group DNA replication persists in the normally arrested epidermis.

DNA replication was assayed by BrdU and shown for 10.5-hr embryos labeled for 40-min followed by antibody staining detected by the horseradish peroxidase (HRP) reaction. The darkly staining cells are those that incorporated BrdU during the pulse.

Panels A and B are at the same magnification and are shown below (A' and B') at a higher magnification. The focus is on the epidermis.

(A-A') BrdU incorporation is shown in a wild-type embryo. Note that BrdU labeling fills the entire nucleus.

(B-B') Inappropriate BrdU incorporation is observed in the epidermis of *l(2)51Ec* homozygous mutant embryos. BrdU labeling is not uniform throughout the nucleus. Note that the labeling is greatly diminished as compared to wild type except for an intensely staining region (arrow) of the nucleus. This punctate pattern of BrdU incorporation is indicative of late replication. DNA replication in the endodomains is mostly blocked.

Discussion

The *in situ* hybridization screen, although labor intensive, has proved to be very successful. The ability to visualize a G1-S transcriptional program in mitotic and endo cycle cells allowed us to isolate both known and novel functions (Table 2). All predicted classes of mutants were identified by this approach. In addition, the types of mutants that were isolated testifies to the specificity of the screen. The screen on the second chromosome was comprehensive. Nearly 9000 lethal mutations (3010 X 2.7) were examined on the second chromosome. The completeness of the screen is also suggested by the number of alleles that compose each complementation group: five for *cyclin E*, five for *dDP*, four for *l(2)51Ec*. There are two loci (*dDP* and novel) that are needed for G1-S transcription in mitotic and polytene cells. All mutations that result in a failure to downregulate the G1-S transcriptional program in endodomains define a single complementation group (*cyclin E*). One gene, *l(2)51Ec*, becomes essential for replication during cycles 14-16. It is possible that we identified all the genes on the second chromosome that regulate the decision to enter S phase. In contrast, the third chromosome screen was not exhaustive. However, it led to the identification of two very interesting alleles of *dE2F*.

It still remains unclear what is required to break the dependence of S phase and mitosis in polytene cells. A mutation specifically affecting the transcription of *PCNA* in all polytene tissues was not recovered on the second chromosome. It is likely, therefore, that a global control necessary for the onset of polytenization is not found on the second chromosome or its function is supplied by maternal contributions of wild-type product. Two mutations on the third chromosome reduce *PCNA* transcription specifically in endo cycle cells. Both mutations are in the *dE2F* gene. Further analyses have shown that these new mutations may not be strictly endo cycle specific although most of the defects are observed in polyploid cells (Chapter 4).

Table 2: Summary of mutants from *in situ* hybridization screen

	second chromosome	third chromosome
Postblastoderm mutants:		
l(2)51Ec	4 alleles	
pimples	2 alleles	
three rows	1 allele	
four novel genes	1 allele/each	
one novel gene		1 allele
Morphologically normal mutants:		
dDP	5 alleles	
one novel(*3.47*) gene	2 alleles	
cyclin E	5 alleles	
dE2F		2 alleles
one novel gene		1 allele

A global regulator of the endo cell cycle may be identified from a more complete screen on the third chromosome and/or a similar screen on the X chromosome. It is also possible that the downregulation of mitotic functions is sufficient to convert a mitotic cycle into an endo cell cycle. If that is the case, a mutation in a gene that controls the switch to an endo cell cycle would result in ectopic mitotic events (e.g. spindle formation and condensed chromosomes) in cells that are normally polytene. It is not clear that the presence of ectopic spindles would disrupt the G1-S transcriptional program. Therefore, loss of a critical endo-specific function may not be detected by our approach.

The mutants identified in this screen allow the *in vivo* role of known cell cycle regulators to be evaluated and provide insights into the regulation of replication in polytene and mitotic cells. In addition to the mutations in *dDP*, a novel complementation group on the second chromosome appears to be required for the induction of G1-S transcription in mitotic and polytene tissues after mitosis 16. This complementation group may turn out to regulate the G1-S transition. This group needs to be mapped genetically, and the cytological location can then be determined by performing complementation tests with second chromosome deficiencies. Deficiencies can also be used to assess whether the mutations are hypomorphs or nulls. The mutations are likely to be weak, because the levels of S phase transcripts were not dramatically reduced in either of the mutant lines. Further phenotypic analysis can be pursued once a deficiency or a strong allele is isolated.

l(2)51Ec becomes limiting for replication during the postblastoderm divisions. Further characterization and the cloning of *l(2)51Ec* will help to determine the role of this gene in replication. At this point, it is not clear whether the failure to downregulate G1-S transcription in the epidermis is the cause or the consequence of the replication defect during cycles 14-16. It is possible that in the *l(2)51Ec* mutants, existing S phase transcripts are stabilized or constitutively transcribed due to a

failure to progress through S phase. Thus, persisting transcripts may be a secondary consequence of the S phase arrest. A similar situation may occur in the *cyclin E* mutants. In the *cyclin E* mutants, S phase is blocked after cycle 16 (Knoblich, et al. 1994). S phase genes including *cyclin E* are transcribed in the normal spatio-temporal pattern but then persist inappropriately (Duronio and O'Farrell 1995; Sauer, et al. 1995). It has been suggested that *cyclin E* is actively involved in down-regulating its own transcription as well as that of other S phase genes (*PCNA* and *RNR2*). However, it is possible that the block to S phase progression in *cyclin E* mutants may be responsible for the failure to extinguish the expression of S phase genes. It is clear both from *l(2)51Ec* and the *cyclin E* mutants that the activation of S phase genes in the correct developmental pattern is not dependent on S phase. Thus, if the decision to progress from G1 into S phase is reflected by the associated G1-S transcriptional program, then *l(2)51Ec* and *cyclin E* act downstream of these events.

The new mitotic mutants isolated in this screen may also prove to be very interesting. The new genes are likely to provide further insight into the regulation of the metaphase/anaphase transition in mitotic cells. In addition, functions that promote exit from mitosis may also be involved in repressing mitotic activities in endo cycle cells.

Materials and Methods

Fly strains

The *cn bw sp* and *DTS91* stocks were provided by R. Lehmann (Skirball Institute, New York University Medical Center, New York, NY). The *Ubx-lacZ CyO* chromosome has been described previously (McCall, et al. 1994).

EMS mutagenesis and genetic screen

See Figure 1 for an outline of the screen. Isogenized *cn bw sp* males were mutagenized with a 35mM EMS solution (Sigma) in 1% sucrose for 24 hours. The males were then mated to *DTS91 pr cn / P[Ubx-lacZ] CyO* virgin females. Male progeny from this cross were mated singly to *DTS91 pr cn / P[Ubx-lacZ] CyO* virgin females. The dominant temperature-sensitive lethal mutation (*DTS91*) chromosome was used to select for flies bearing the mutagenized chromosome over the *P[Ubx-lacZ] CyO* balancer. The crosses were placed at 29°C, the restrictive temperature for the *DTS91* mutation, for three days. Then, the adults were removed, and the vials were incubated at 29°C for an additional day before being moved to 25°C. *cn bw sp / P[Ubx-lacZ] CyO* females and males from this cross were selected and mated to establish mutant lines. 8-15 hour embryos were collected from these lines and hybridized simultaneously with *PCNA* and *lacZ* riboprobes.

In situ hybridization procedure

In situ hybridizations were performed on 144 independent mutagenized lines at a time. Embryos were collected from mutant lines using the block method (Nüsslein-Volhard, et al. 1984) and staged appropriately (8-15hr). To obtain eggs from 144 mutant lines eight collection blocks (18 lines each, 10-15 flies per line) were set up before each experiment. It usually took 2-3 days for the flies to adjust to the

collection blocks and to begin to lay eggs well. Three collections from each block were needed to obtain enough embryos from each line to score for the affect on *PCNA* expression. The collections were carried out over a period of three days. The eggs from the first and second day where kept at 4°C until the last collection was ready. Then eggs were transferred from the apple juice agar plates into four 36-well staining dishes. Each well had three sets of egg collections from a single mutagenized line.

All the steps to prepare embryos for *in situ* hybridization were carried out in the 36-well dishes. The embryos were washed in water three times, dechorionated for 5 min in 50% bleach, washed three times in water, and fixed with gentle shaking in 1:1 heptane:12% paraformaldehyde in PBS. Embryos were then washed twice in heptane briefly. To devitellinize the embryos an equal volume of heptane and methanol were added followed by vigorous shaking for four minutes. The embryos were then washed in methanol several times to remove all traces of heptane. The devitellinized embryos sank to the bottom and the non-devitellinized embryos were removed from the top by aspiration. The remaining embryos were rehydrated and subject to *in situ* hybridization.

Digoxygenin-labeled antisense riboprobes were made according to the Boehringer Mannheim kit. *In situ* hybridization was carried out essentially as described (Tautz and Pfeifle 1989) with a few key modifications to reduce the levels of background due to the use of the multi-well staining dishes. The fixation after the proteinase K treatment was done for 20 min in 8% paraformaldehyde in PBS. Hybridization was carried out at 65°C. 1% sheep serum was added to incubation with the anti-digoxygenin antibody conjugate (Boehringer Mannheim) used at 1:4000 in PBT (PBS/.05% Tween 20). After the embryos were stained, the embryos from each well were transferred into an eppendorf tube and mounted in 70% Glycerol: 30% PBS. Each line was then examined microscopically for changes in the *PCNA* transcription pattern.

BrdU labeling of embryos

Embryos were collected from mutagenized stocks for seven hours at 25°C and aged for 12 hours at 18°C (this is equivalent to 8 hours at 25°C). The resulting 8-15hr collection of embryos was then permeabilized with octane and labeled with BrdU. These and subsequent steps were carried out essentially as described previously (Bodmer, et al. 1989). The embryos were incubated with BrdU for 40 min. at room temperature while shaking in a scintillation vial. After fixation, the embryos were hydrolyzed in 2N HCl for 70 min. The length of the HCl treatment is important for good detection of BrdU incorporation. An anti-*B*-galactosidase antibody was used along with the anti-BrdU antibody. The anti- *B*-galactosidase antibody allows the 25% homozygous mutant embryos to be distinguished from their siblings (the heterozygotes express *lacZ* from the *Ubx-lacZ* transgene on the balancer chromosome). The antibody staining was detected using the horseradish peroxidase histochemical reaction.

Microscropy

A Zeiss Axiophot microscope equipped with Nomarski optics was used to examine and photograph the embryos using Plan-Neofluar 10X, 20X, and 40X objectives.

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Chapter Three

Mutations in Drosophila *DP* and *E2F* distinguish G1-S progression from an associated transcriptional program

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Abstract

The E2F transcription factor, a heterodimer of E2F and DP subunits, is capable of driving the G1-S transition of the cell cycle. However, mice in which the *E2F-1* gene had been disrupted developed tumors, suggesting a negative role for E2F in controlling cell proliferation in some tissues. The consequences of disrupting the *DP* genes have not been reported. We screened for mutations that disrupt G1-S transcription late in Drosophila embryogenesis and identified five mutations in the *dDP* gene. Although mutations in *dDP* or *dE2F* nearly eliminate E2F-dependent G1-S transcription, S phase still occurs. Cyclin E has been shown to be essential for S phase in late embryogenesis, but in *dDP* and *dE2F* mutants the peaks of G1-S transcription of *cyclin E* are missing. Thus greatly reduced levels of *cyclin E* transcript suffice for DNA replication until late in development. Both *dDP* and *dE2F* are necessary for viability, and mutations in the genes cause lethality at the late larval/pupal stage. The mutant phenotypes reveal that both genes promote progression of the cell cycle.

Introduction

The regulation of the transition between the G1 gap phase and S phase is a critical cell cycle control point, because duplication of the DNA commits a cell to division. This transition has been inferred to be controlled both at the posttranscriptional and transcriptional level (Murray and Hunt 1993; Nasymth, 1996). In the yeast *S. cerevisiae* the activation of the CLN/CDC28 kinase complex drives the cells into S phase, while in mammalian cells activation of the cyclinE/CDK2 complex is crucial for the onset of DNA replication. Thus phosphorylation of key substrates by these kinase complexes is one regulatory component of the initiation of S phase. Regulation also appears to occur at the level of transcription, however, because *CLN 1* and *2* and *cyclin E* transcripts accumulate in G1. In addition, many genes encoding proteins necessary for DNA replication are transcribed at the onset of S phase.

E2F is a transcription factor whose activity has been linked to the G1-S transition in mammalian cells. This transcription factor is a heterodimer of the E2F protein and the DP protein, and there are several forms of either protein present in mammalian cells (Weinberg 1995). E2F/DP has been shown to activate the transcription of several genes needed for S phase, and E2F/DP binding sites are found in the promoters of many others (Nevins 1992). In addition, E2F/DP binding may repress the transcription of some of these genes during G1 when the heterodimer is complexed with one of the family of pocket proteins, one member of which is pRB (Weintraub, et al. 1995; Weintraub, et al. 1992; Zwicker, et al. 1996). E2F/DP is the focal point of a regulatory loop that links both transcriptional and posttranscriptional control of the cell cycle (for review see Sherr, 1996). Phosphorylation of pRB by either the cyclinD/CDK4,6, cyclinE/CDK2, or cyclinA/ CDK2 kinases releases pRB, possibly enabling E2F/DP to function as a transcriptional activator. Because the

cyclin E gene itself is transcriptionally controlled by E2F/DP, a positive feedback loop ensues (Botz, et al. 1996; Geng, et al. 1996; Ohtani, et al. 1995).

It is not clear whether *in vivo* E2F/DP acts primarily as a positive or negative transcription factor or whether it plays alternate predominant roles in different types of tissues. In addition, while the importance of transcriptional control of the onset of S phase is inferred from its correlation with DNA replication, its significance relative to posttranscriptional control mechanisms is not established. Overexpression of E2F protein in mammalian cell culture drives cells into S phase (Johnson, et al. 1993; Lukas, et al. 1996; Shan and Lee 1994). However, transgenic mice lacking a functional E2F-1 gene exhibited phenotypes consistent with the E2F transcription factor acting either positively or negatively in different tissues (Field, et al. 1996; Yamasaki, et al. 1996). In these mice some tissues atrophy, but other tissues develop tumors. The consequences of disrupting the DP genes have not been reported.

Drosophila shares many of the components of the mammalian G1-S regulatory circuitry. Drosophila homologs to E2F, DP, an RB-like protein (RBF), cyclin E, CDK2, cyclin D, CDK4, and the p21 inhibitory protein (*dacapo*) have been identified (de Nooij, et al. 1996; Du, et al. 1996a; Dynlacht, et al. 1994; Finley, et al. 1996; Hao, et al. 1995; Knoblich, et al. 1994; Lane, et al. 1996; Ohtani and Nevins 1994; Richardson, et al. 1993; Sauer, et al. 1995; Sauer, et al. 1996). The precise developmental control of the onset of S phase late in Drosophila embryogenesis makes it possible to define mutant phenotypes with high resolution and to infer the primary defect in cell cycle regulation (Smith, et al. 1993; Smith and Orr-Weaver 1991). The first 13 divisions in the embryo occur in a rapid S-M cycle that is driven by maternal stockpiles of cell cycle regulators (for reviews see Foe, et al. 1993; Orr-Weaver 1994). Following cellularization and the onset of zygotic gene expression, another three cell cycles take place with a G2 phase during which transcription

occurs, but they lack a G1 phase. After mitosis 16, a detectable G1 phase appears for the first time during embryogenesis, and it marks the onset of the endo cell cycle for many of the larval tissues. In these cells S phase alternates with a gap phase, but mitosis does not occur, leading to polyteny. During the latter half of embryogenesis the cells that will form the polytene larval tissues undergo S phase in a stereotypic developmental pattern. During the same developmental period the nervous system cells continue to go through mitotic divisions.

Cyclin E is essential for S phase late in Drosophila embryogenesis (Knoblich, et al. 1994; Sauer, et al. 1995). Mutations in *cyclin E* block DNA replication after mitosis 16, coincident with the first G1 phase. Presumably cyclin E is necessary for S phase earlier in embryogenesis but maternal stockpiles of the protein and/or transcripts suffice (Richardson, et al. 1993). The onset of the requirement for cyclin E correlates with a change in its transcriptional regulation (Knoblich, et al. 1994). During the postblastoderm divisions the levels of *cyclin E* transcript are constitutively high. After mitosis 16 they are downregulated in all of the tissues except the nervous system, and transcripts accumulate prior to S phase in the endo cycle late in embryogenesis. The same transcriptional pattern observed for *cyclin E* also occurs for several genes encoding replication functions: *PCNA*, *RNR1*, *RNR2*, and *pol* α (Duronio and O'Farrell 1994). These observations led to a model in which transcriptional regulation is crucial in controlling the G1-S transition in the endo cycle, with *cyclin E* being the critical target (Duronio and O'Farrell 1994; Duronio and O'Farrell 1995; Sauer, et al. 1995).

In order to identify regulatory genes needed for G1-S transcription, we screened for mutants defective in the induction of *PCNA* transcription late in embryogenesis. We found five mutations in a gene essential for *PCNA* and *RNR2* transcription and demonstrated that these are alleles of the Drosophila *DP* gene. We show that the *dDP* gene is essential for viability. Despite the pronounced effect on G1-S

transcription, DNA replication still occurs and lethality is late in development. We find that mutations in *dE2F* give similar phenotypes to those in *dDP*: E2F/DP-dependent G1-S transcription is disrupted, but S phase takes place and the animals survive to late larval/ early pupal stages.

Results

Screen for genes required for S Phase transcription in the embryo

To recover genes necessary for the transcription of S phase genes late in Drosophila embryogenesis, we directly used the trancription pattern as the assay in a genetic screen. The *PCNA* and *RNR2* genes were used because they are transcribed in a pattern that mimicks that observed by BrdU labeling of late Drosophila embryos (Duronio and O'Farrell 1994). The E2F/DP recognition sites in the *PCNA* promoter are essential for its expression (Yamaguchi, et al. 1995). We devised a method for *in situ* hybridization to detect transcripts on large numbers of independent mutagenized lines. Embryos were collected from lines established from single, mutagenized second chromosomes, aged to 8-15 hours, and hybridized *in situ* to a *PCNA* riboprobe (see Methods, Fig. 7). This developmental stage was chosen because between 8-15 hours there is a G1 phase with a regulated transcriptional program. In addition, S phase transcription can be observed both in cells undergoing endo cycles as well as in the mitotically dividing cells of the nervous system.

From 3010 mutated second chromosome lines, seven lines were recovered that failed to express the *PCNA* transcript at normal levels in 8-15 hour embryos. In all of these lines *PCNA* transcript was reduced in both the mitotic and endo cycle tissues. As a secondary test, the *RNR2* transcript was also affected in the mutant embryos. Complementation tests showed that the mutants fell into two complementation groups. One group, containing two alleles, showed a reduction in *PCNA* and *RNR2* transcript levels. In the second group of five mutations had a stronger reduction in *PCNA* or *RNR2* transcripts to nearly undetectable levels late in embryogenesis. This latter complementation group was subjected to further analysis.

Mutations in the Drosophila *DP* gene

The expression of *PCNA* and *RNR2* in the strong complementation group was almost identical to that previously described for mutations in the *dE2F* gene (Fig. 1) (Duronio, et al. 1995). The one distinction was that in the new complementation group there was a higher level of *RNR2* transcript uniformly present throughout the epidermis. This was a weak signal that gave the appearance of a higher background in the homozygous mutant embryos (data not shown).

A homolog to the mammalian DP protein, the other subunit of the E2F transcription factor, was identified in Drosophila (*dDP*) and mapped to the second chromosome. Mutations in the *dDP* gene had not been identified (Hao, et al. 1995). Given the similarity between the *dE2F* phenotype and that of our mutants, we tested whether a deficiency known to delete the *dDP* gene also uncovered our complementation group. All five mutations were lethal *in trans* to each of the three deletions that remove *dDP*. Furthermore, *in situ* hybridization of embryos transheterozygous for the mutation and the *Df(2R)vg-B* deficiency gave the mutant phenotype, undetectable *PCNA* expression in late embryos.

To confirm that the mutations mapped to the *dDP* gene, we sequenced the *dDP* gene from two of the mutant lines. Because the mutations cause pupal lethality (see below) we were able to isolate genomic DNA from larvae transheterozygous for the mutation and a deficiency. A region of 1400 base pairs that encompasses the regions of the protein conserved with mammals was amplified by PCR from mutant larval genomic DNA, and the PCR product was sequenced directly. The sequenced region contains several important motifs, including the DNA binding region, the DEF box which is predicted to be required for DP/E2F heterodimerization, and three other highly homologous regions named DCB1, DCB2, and NCB (Fig. 2) (Dynlacht, et al. 1994; Hao, et al. 1995). To recognize polymorphisms between our strains and those used for the published sequence, we also sequenced the same region from

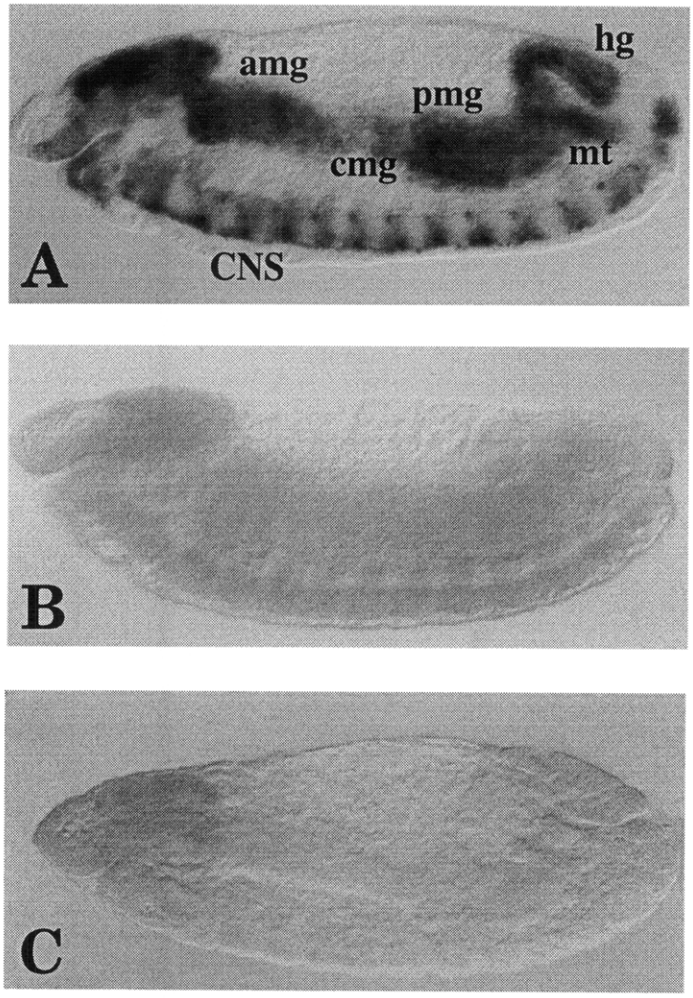


Figure 1. *PCNA* expression in *dDP*and *dE2F* mutant embryos.

PCNA transcripts were detected by whole-mount in situ hybridization.

(A) In wild-type 10.5 hour embryos *PCNA* is expressed in the mitotically proliferating central nervous system (CNS), in the endoreplicating anterior midgut (amg), central midgut (cmg), posterior midgut (pmg), hindgut (hg), and malpighian tubules (mt).

(B) In 10.5 hour homozygous dDP^{a4} mutant embryos *PCNA* is expressed at greatly reduced levels in the CNS and is not detectable in the endodomains.

(C) $dE2F^{91}$ homozygous 10.5 hour embryos show a similar phenotype. In some embryos a low level of *PCNA* expression can be seen in the CNS, but expression is absent in the endodomains.

transheterozygous adults from two unrelated lines recovered from the screen. Within the region we sequenced there are six introns ranging in size from 56 to 68 base pairs (Fig. 2). There is a polymorphism in our strains, changing the histidine at position 275 to a leucine. This histidine residue is conserved between humans, mice, and Xenopus and is present in the DCB2 box (Dynlacht, et al. 1994; Hao, et al. 1995). However, it is not essential given that strains containing a leucine at this position have a wild-type phenotype .

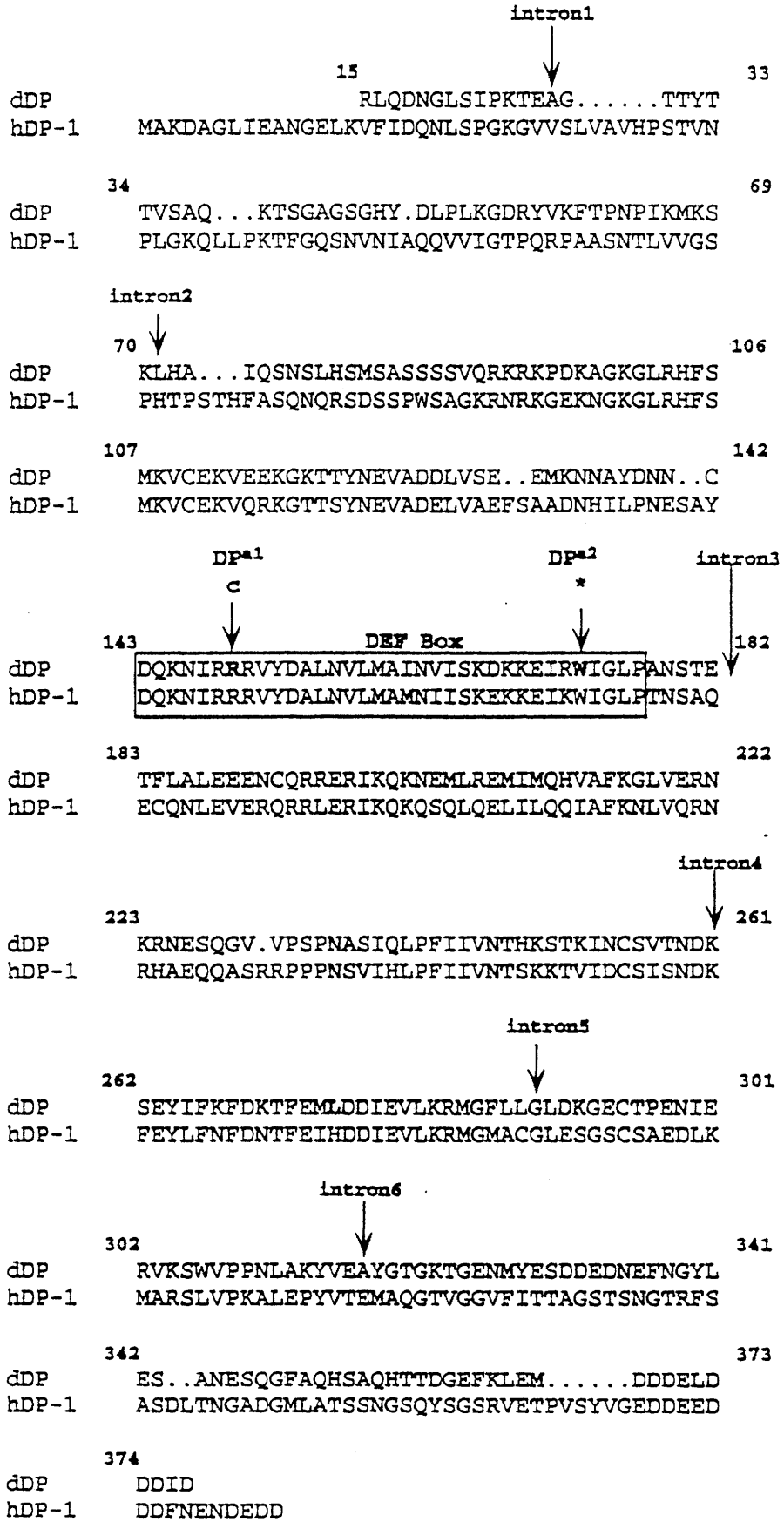
We found that both of the mutations were associated with codon changes within the *dDP* open reading frame (Fig. 2). The first mutation is a C to T nucleotide transition that changes Arg^{149} to a Cys. This residue lies within the DEF box in a block of amino acids that is highly conserved between DP and E2F, and Arg^{149} is conserved between Drosophila, mice, and humans (Dynlacht, et al. 1994; Hao, et al. 1995). With sequence confirmation that the mutation disrupted the *dDP* ORF, this allele was named dDP^{a1}. The second mutation is a G to A nucleotide transition that converts Trp^{173} to a stop codon. The resulting truncated protein would lack part of the DEF box as well as the three highly conserved domains, DCB1, DCB2, and NCB. This allele was named dDP^{a2}. The molecular data combined with the genetic complementation data on the five mutations confirm that this complementation group is the Drosophila *dDP* gene. Consequently, the other mutations have been designated dDP^{a3}, dDP^{a4}, and dDP^{a5}.

The role of *dDP* and *dE2F* in G1-S progression

In mammalian cells, the E2F transcription factor triggers the expression of essential S phase genes, and the transcription of these genes correlates with progression into S phase. In Drosophila, mutations in the *dE2F* gene were reported to block the G1-S transition and eliminate detectable DNA replication late in

Figure 2. Amino acid changes in the *dDP*^{a1} and *dDP*^{a2} mutant lines.

The wild-type Drosophila *dDP* sequence from a 1400 base pair region PCR amplified from genomic DNA is compared with the human DP-1 sequence. The amino acid numbering is that of Dynlacht et al. (Dynlacht, et al. 1994). Amino acid changes in *dDP*^{a1} and *dDP*^{a2} are indicated, and both of these changes occur in the DEF box (outlined). The region of genomic DNA sequenced contains six small introns: the first, in amino acid 28, is 57 bp; the second, in amino acid 71, is 63 bp; the third, after amino acid 182, is 68 bp; the fourth, in amino acid 261, is 60 bp; the fifth, in amino acid 289, is 63 bp; and the sixth, in amino acid 317, is 56 bp. The sequence shown does not include the first 14 amino acids of the N terminus reported by Dynlacht et al. (1994). The polymorphism is present between our sequence and that of Dynlacht et al., is shown in bold.


intron1
15
33
dDP RLQDNGLSIPKTEAG.....TTYT
hDP-1 MAKDAGLIEANGELKVFIDQNLSPGKGVVSLVAVHPSTVN
34
69
dDP TVSAQ...KTSGAGSGHY.DLPLKGDRYVKFTPNPIKMKS
hDP-1 PLGKQLLPKTFGQSNVNIAQQVVIGTPQRPAASNTLVVGS
intron2
70
106
dDP KLHA...IQSNSLHSMSASSSSVQRKRKPDKAGKGLRHFS
hDP-1 PHTPSTHFASQNQRSDSSPWSAGKRNRKGEKNGKGLRHFS
107
142
dDP MKVCEKVEEKGKTTYNEVADDLVSE..EMKNNAYDNN..C
hDP-1 MKVCEKVQRKGTTSYNEVADELVAEFSAADNHILPNESAY
DPa1
C
DPa2
*
intron3
143
DEF Box
182
dDP DQKNIRRRVYDALNVLMAINVISKDKKEIRWIGLPANSTE
hDP-1 DQKNIRRRVYDALNVLMAMNIISKEKKEIKWIGLPTNSAQ
183
222
dDP TFLALEEENCQRRERIKQKNEMLREMIMQHVAFKGLVERN
hDP-1 ECQNLEVERQRRLERIKQKQSQLQELILQQIAFKNLVQRN
intron4
223
261
dDP KRNESQGV.VPSPNASIQLPFIIVNTHKSTKINCSVTNDK
hDP-1 RHAEQQASRRPPPNSVIHLPFIIVNTSKKTVIDCSISNDK
intron5
262
301
dDP SEYIFKFDKTFEMLDDIEVLKRMGFLLGLDKGECTPENIE
hDP-1 FEYLFNFDNTFEIHDDIEVLKRMGMACGLESGSCSAEDLK
intron6
302
341
dDP RVKSWVPPNLAKYVEAYGTGKTGENMYESDDEDNEFNGYL
hDP-1 MARSLVPKALEPYVTEMAQGTVGGVFITTAGSTSNGTRFS
342
373
dDP ES..ANESQGFAQHSAQHTTDGEFKLEM.....DDDELD
hDP-1 ASDLTNGADGMLATSSNGSQYSGSRVETPVSYVGEDDEED
374
dDP DDID
hDP-1 DDFNENDEDD

embryogenesis (Duronio, et al. 1995). We tested whether the mutations in *dDP* also would block entry into S phase.

To determine if *dDP* is required for G1-S progression, we analyzed BrdU incorporation after the first G1 phase in Drosophila embryogenesis. Normally this G1 phase is followed by polytene replication in an invariant tissue-specific pattern in the embryo (Fig. 3A) (Smith and Orr-Weaver 1991). 8 - 15 hour embryos were collected from *dDP* mutant lines and labeled with BrdU during a 40 minute interval. To our surprise we detected BrdU incorporation in embryos homozygous for mutant alleles of *dDP*, and the spatio-temporal pattern of BrdU incorporation in these embryos was normal (Fig. 3B).

The normal replication observed in the *dDP* mutants prompted us to examine DNA synthesis in the $dE2F^{91}$ null allele as well as the $dE2F^{7172}$ mutant. The $dE2F^{91}$ mutation is a stop codon early in the dE2F coding sequence (Duronio, et al. 1995). The $dE2F^{7172}$ mutation is also likely to be a null because dE2F protein is not detectable in homozygous embryos (Asano, et al. 1996). Furthermore, the failure to observe protein in these mutant embryos implies that maternal pools of the E2F protein do not persist in late embryos. Strikingly, DNA replication was observed in embryos mutant for either of the *dE2F* alleles (Fig. 3C, D), although the intensity of BrdU incorporation was slightly diminished. As a control, we repeated the 40 minute BrdU pulse on embryos homozygous for *cyclin* E^{PZ5}. As expected, BrdU incorporation was not detectable in the polytene tissues of *cyclin E* mutant embryos (Fig. 3H, arrows). We conclude that even though the bursts of E2F-dependent G1-S transcription are not evident in *dDP* and *dE2F* mutant embryos, DNA replication still occurs.

Although DNA replication takes place in *dDP* and *dE2F* mutant embryos, two observations suggest that the rate of replication is slowed. First, if a 10 minute pulse of BrdU labeling was used, BrdU incorporation was reduced in both polytene and

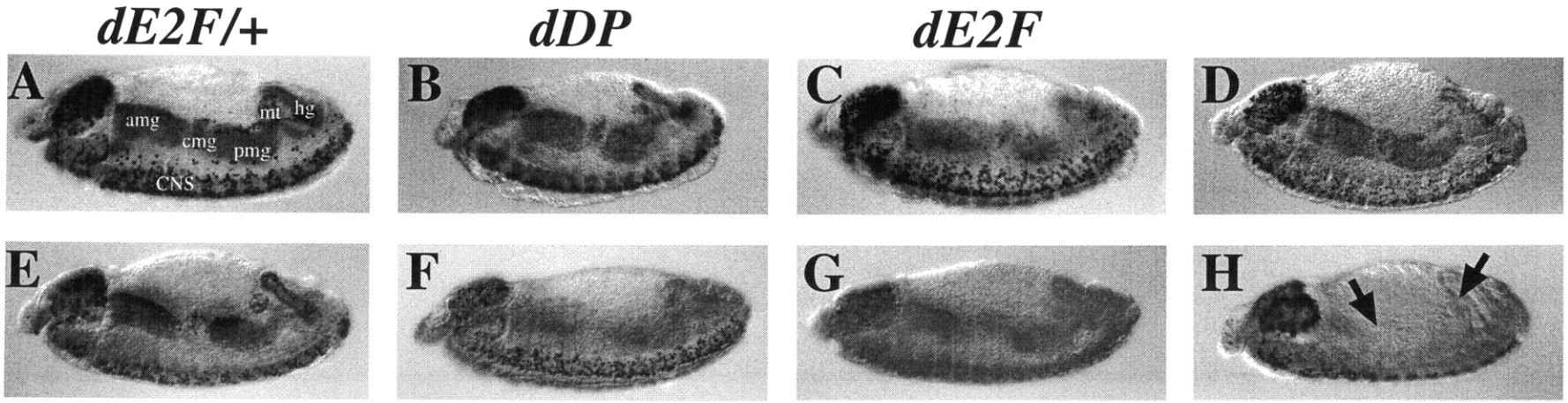


Figure 3. S phase occurs in *dDP* and *dE2F* mutants.

DNA replication was assayed by BrdU labeling of 10.5 hour embryos for either 40 minutes (A-C, H), 20 minutes (D), or 10 minutes (E-G) followed by antibody staining detected by the HRP reaction. The darkly staining cells are those that incorporated BrdU during the pulse. The central nervous system (CNS), anterior midgut (amg), central midgut (cmg), posterior midgut (pmg), malpighian tubules (mt), and hindgut (hg) incorporate BrdU.

The characteristic replication pattern can be seen for the 40 minute BrdU pulse for (A) $dE2F^{7172}$/+ embryos which have a wild-type phenotype, homozygous mutant (B) dDP^{a4} and (C) $dE2F^{91}$ embryos, (D) for the 20 minute pulse in the homozygous $dE2F^{7172}$, and (E) for the 10 min BrdU pulse for $dE2F^{7172}$/+ embryos. The 10 minute BrdU pulse for the dDP^{a4} and $dE2F^{91}$ mutants shows reduced labeling in both the mitotic (CNS) and polytene cells (F, G), but replication is still detected. (H) There is no detectable replication in the endodomains (arrows) of a *cyclin* E^{PZ5} homozygous mutant after a 40 minute BrdU pulse.

neural tissues of homozygous *dE2F* or *dDP* embryos (Fig. 3F, G), while replication was normal with this short pulse in heterozygous controls (Fig. 3E). The previous studies that concluded DNA replication was undetectable in *dE2F* mutant embryos used a short pulse of BrdU labeling (Duronio et al. 1995). However, even with a 10 minute BrdU pulse the level of labeling in *dE2F* and *dDP* mutants is much higher than that obtained in the endodomains with a 40 minute pulse of the *cyclin E* mutant (Fig. 3F, G, H). Second, in the *dE2F* mutants the developmental time of the onset of the later polytene S phases appeared delayed. Replication persisted in the anterior and posterior midgut at a developmental stage when only the central portion of the midgut normally replicates. In the mutant embryos the second round of polytene replication in the central midgut was not observed (data not shown), suggesting it was delayed until after cuticle deposition when antibody detection is no longer possible.

Developmental phenotypes of *dE2F* and *dDP*

To evaluate whether the E2F/DP transcription factor functions to promote entry into S phase and/or suppress hyperproliferation, we determined the furthest developmental point reached by analyzing the lethal phase of both the *dE2F* and the *dDP* mutants. Since S phase occurs during embryogenesis in *dDP* and *dE2F* mutants, we did not expect the mutations to cause embryonic lethality, although the *dE2F* mutations were previously described as being embryonic lethal (Duronio, et al. 1995). We tested whether the *dDP* and *dE2F* mutants were embryonic lethal by scoring whether the embryos hatched as first instar larvae. For all five of the *dDP* alleles heterozygous mutant/+ females were crossed to *Df*/+ males. At least 300 eggs were collected and scored, but no embryonic lethality of the *dDP* alleles *in trans* to the deficiency was observed. In the same way, we tested the $dE2F^{91}$ and $dE2F^{7172}$ alleles *in trans* to a deficiency that uncovers *dE2F*. The alleles were also examined *in trans* to each other. In each experiment 500 eggs were collected and

scored, and there was no embryonic lethality. We tested $dE2F^{91}$ homozygous embryos and found the homozygous chromosome to cause embryonic lethality. However, this is clearly the result of other mutations on the chromosome, since both the $dE2F^{91}$ and $dE2F^{7172}$ *in trans* to a deficiency or each other are not embryonic lethal.

We examined the role of *dE2F* in larval and pupal development in order to determine whether the gene was essential and whether the *dE2F* mutations caused any defects in polytenization or cell proliferation. In Drosophila most of the larval cells do not undergo mitosis after completion of embryogenesis but grow from polytene replication. However, the imaginal cells that will form the adult body remain diploid. Thus defects in the endo cell cycle producing polytene cells would result in small larvae, while defects in proliferation would be manifest after pupation when the imaginal cells differentiate to produce the adult body. Larvae and pupae mutant for *dE2F* were identified by the absence of a dominant marker (see Methods). Approximately 240 total larvae were examined, and 63% of the $dE2F^{91}/dE2F^{7172}$ survived to third instar. 54% of these third instar larvae initiated pupation. Because the mutant animals die as late larvae or early pupae, *dE2F* is clearly essential for development. The late lethality of the *dE2F* mutant is consistent with the high levels of *dE2F* protein observed in wild-type second and third instar larval extracts (Brook, et al. 1996).

Although the *dE2F* mutant animals survive through larval life, we observed a dramatic delay in larval growth. It took between 288-432 hours for the *dE2F* mutant larvae to pupate, compared to 120 hours for heterozygous sibling controls. Five days after egg laying the *dE2F* mutant larvae were very sluggish and much smaller in size than their wild-type counterparts (Fig. 4A, B). The polytene salivary gland and diploid imaginal discs could not be identified in the five day old *dE2F* mutant larvae, presumably because they were so small. The brains were also greatly reduced in size

as compared to wild-type (Fig. 4 E, F). The size of the *dE2F* mutant larvae increases over time (Fig. 4B, C), and the internal tissues approached wild-type size (Fig. 4F, G and data not shown). DNA replication therefore can occur during this larval period, but it is slow. Replication in the absence of dE2F is further evidenced by the formation of banded polytene salivary gland chromosomes in some of the 12-18 day larvae (Fig. 4H). Although the polytene chromosomes from the *dE2F* mutant larvae were smaller and more fragile than normal they were clearly visible. Thus, we conclude that S phase occurs in the absence of *dE2F*, but *dE2F* is necessary for timely replication and growth.

In addition to the growth delay, the *dE2F* mutant larvae had another striking phenotype, melanotic pseudotumors were formed (Fig. 4C, D). Melanotic tumors are groups of cells within the larvae that are recognized by the immune system and encapsulated in melanized cuticle (Sparrow 1978; Watson, et al. 1991). We refer to them as pseudotumors to emphasize that they are not necessarily the consequence of hyperproliferation but can be abnormal cells recognized by the immune cells. Small pseudotumors were first observed in the *dE2F* mutants seven days after egg laying, and these early pseudotumors grew and darkened as the larvae aged. In the *dE2F* mutants that initiated pupation, numerous additional small pseudotumors formed.

We compared the lethal phenotype of the *dDP* alleles over a deficiency to that of the *dE2F* mutations (Table 1). Unlike the *dE2F* mutants, the larval growth of the *dDP* mutants was not dramatically delayed. The lethality was largely pupal, and some melanotic pseudotumors were observed in the early pupae. The *dDP* alleles ranged in the severity of the phenotype they produced (Table 1). The strongest phenotype was observed with dDP^{a5} which resulted in late larval lethality when *in trans* to a deficiency. The dDP^{a2} and dDP^{a4} mutants *in trans* to a deficiency survived to the pupal stage. We think that the pupal stage is the lethal phase, and

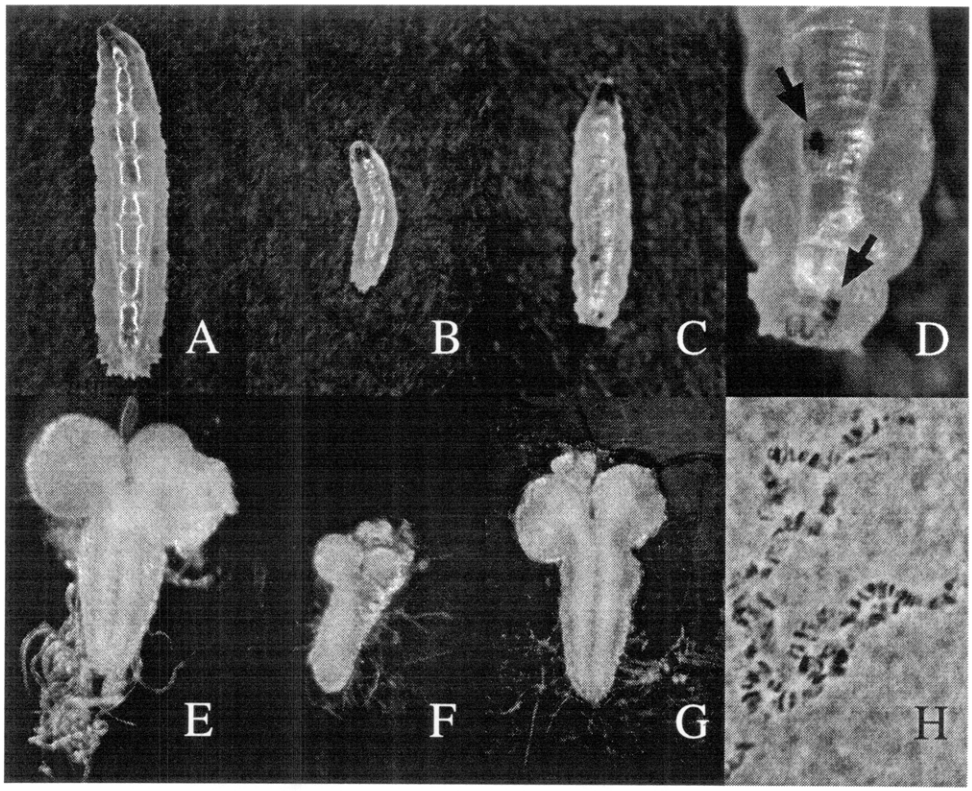


Figure 4. *dE2F* is required for wild-type growth in mitotic and polytene tissues.

(A-C) All the larvae are at the same magnification.

(A) $dE2F^{7172}$/+ larva at 5 days AEL (after egg laying).

(B) $dE2F^{91}/dE2F^{7172}$ mutant larvae 5 days AEL.

(C) $dE2F^{91}/dE2F^{7172}$ mutant larva 13 days AEL with two posterior melanotic pseudotumors.

(D) a magnification of posterior pseudotumors (arrows) shown in C.

(E-G) All the brains are at the same magnification.

(E) brain of $dE2F^{7172}$/+ larva at 5 days AEL.

(F) brain of $dE2F^{91}/dE2F^{7172}$ mutant larva at 5 days AEL.

(G) brain of $dE2F^{91}/dE2F^{7172}$ mutant larva at 9 days AEL.

(H) Polytene chromosomes from the salivary glands of $dE2F^{91}/dE2F^{7172}$ mutant larva at 13 days AEL.

Table 1. Developmental phenotypes of *dDP* mutants

Genotype	Lethal Phase[a]	Phenotype
dDP^{a1}/Df(2R)vg-B	adult flies 11%-20%; adults in pupal case 80%-89%	rough eyes, incomplete wing vein, thin and short bristles, etched tergites, more female files than males, females and males sterile, females lay eggs with thin chorions
dDP^{a3}/Df(2R)vg-B	adults in pupal case	rough eyes, etched tergites, thin and short bristles
dDP^{a2}/Df(2R)vg-B *dDP^{a4}/Df(2R)vg-B*	adults in pupal case 50%; pupae 50%	kidney-shaped rough eyes, thin and short bristles on thorax and head, severe abdominal defects
dDP^{a5}Df(2R)vg-B	late larval lethal	

[a]Lethal phase is the furthest development stage reached by the *dDP* mutants.

that the dDP^{a5} mutant is unusual. The dDP^{a5} larval lethality is either due to another mutation on the chromosome that enhances the phenotype, or because this allele is antimorphic. The phenotype of the other alleles *in trans* to dDP^{a5} was stronger than *in trans* to the deficiency.

Approximately half of the dDP^{a2}/*Df* pupae reached adulthood in the pupal case (Table 1). These adults struggled to eclose but ultimately died. Organisms dissected from the pupal case had essentially normal heads and thoraxes. However, their abdominal defects were severe. This is informative since the head and thorax are derived from imaginal discs, while the abdomen arises from the abdominal histoblast nests. The imaginal discs proliferate during larval stages, but the abdominal histoblast nests proliferate during pupal development. Thus, pupal lethality may result from a defect in abdomen formation that occurs during pupal development.

The weakest allele, dDP^{a1}, was semilethal *in trans* to a deficiency (Table 1). The recovered adults had rough eyes and wing-vein defects. These phenotypes are diagnostic of compromised mitotic proliferation. They also had thin and short bristles that indicate a defect in polytene replication, because the cells that give rise to the bristle shaft and socket endoreplicate (Lees and Waddington 1942). We conclude that *dDP*, like *dE2F*, provides an essential function for the development of the organism. The eye and bristle defects indicate that *dDP* is required for normal development in both mitotic and endo cycle cells.

Genetic interaction between *dDP* and *dE2F*

To demonstrate that the late lethality and developmental phenotypes associated with the *dDP* mutants were due to loss of dDP function rather than a synthetic effect with other loci, we tested whether the lethality could be rescued by expressing the wild-type *dDP* gene under a heat shock promoter. We introduced an *hsp70-dDP* transgene into the dDP^{a1} background (Table 2) (Duronio, et al. 1996). At

25°C we observed a partial rescue of the dDP^{a1}/Df lethality due to basal expression of *dDP* from the *hsp70* promoter. The recovered dDP^{a1}/Df; *hs-dDP*/+ males were fertile. The recovered adults, however, did have rough eyes, wing vein, and bristle defects and were female sterile. The effect of induced *dDP* expression (37°C heat treatment) on the *dDP* mutants was even more dramatic (Table 2). The lethality of dDP^{a1}/Df was completely rescued, and all the developmental phenotypes were suppressed. The rescue confirms that the pupal lethality, male and female sterility, rough eye, wing vein, and bristle defects are all due to loss of *dDP* function.

Because ectopic expression of *dE2F* and *dDP* in the *Drosophila* eye results in excess cell proliferation (Asano, et al. 1996; Du, et al. 1996b), we examined the effect of *dDP* mutations on eye development in more detail. We used scanning electron microscopy (SEM) to analyze the eyes of dDP^{a1}/dDP^{a2}; *hs-dDP*/+ with and without heat shock. The eyes of *dDP* mutant flies carrying the uninduced *hs-dDP* transgene were extremely rough (Fig. 5B). The reduced size of the eye, missing and disorganized ommatidia, as well as stunted, missing and disorganized bristles suggested a proliferation defect in the *dDP* mutant (Fig. 5B). Strikingly, flies of the same genotype that underwent daily heat shock treatments had normal eyes (Fig. 5A, C). dDP^{a1} *in trans* to a deficiency resulted in the same rough eye phenotype as observed for dDP^{a1}/dDP^{a2} flies at 25°C, and the rough eye was rescued by induced ectopic *dDP* expression (data not shown). Because the dDP^{a2} allele exhibits the same strength phenotype as a deficiency, it genetically behaves as a null allele.

Having shown that heat shock *dDP* rescues the *dDP* mutants we defined the developmental period during which ectopic *dDP* expression is capable of rescuing the lethality of the *dDP* mutants. Females heterozygous for dDP^{a2} were mated to heterozygous dDP^{a1} males carrying the *hs-dDP* transgene. The heat shock regimen began immediately after the 24 hour egg collection was completed or 5 - 6 days later (late larval / early pupal life). Both treatments yielded the same results, 100% rescue

Table 2. Rescue of *dDP* mutants by overexpression of *dDP* and *dE2F*

Genotype of recovered adults	Recovered/expected	
	25°C	37°C
dDP^{a1}/Df; +/+	11%[a] (males and females sterile)	0%[d]
dDP^{a1}/Df; hs-dDP/+	42%[b] (males fertile; females sterile)	100%[e] (males fertile; females semifertile)
dDP^{a1}/Df; hs-dE2F/+	37%[c] (males and females sterile)	18%[f] (males and females sterile)

w; Df(2R)vg56/CyO females were mated to *w; dDP^{a1}/CyO ; P[w$^+$;hs-dDP]/+* or *w; dDP^{a1}/CyO ; P[w$^+$;hs-dE2F]/+* males at 25°C. Eggs collected from the crosses were either allowed to develop at 25°C or received heat shock treatments at 37°C.

The recovered adults of the indicated genotype were expected to be one-quarter of the *CyO* progeny according to Mendelian ratios (see Materials and Methods): [a] 9/324, [b] 34/324, [c] 20/218, [d] 0/184, [e] 51/184 , [f] 12/266. The numerator is the number of recovered adults of the indicated genotype. The denominator is the number of the *CyO* progeny. To calculate the percentage, the ratios were multiplied by 4.

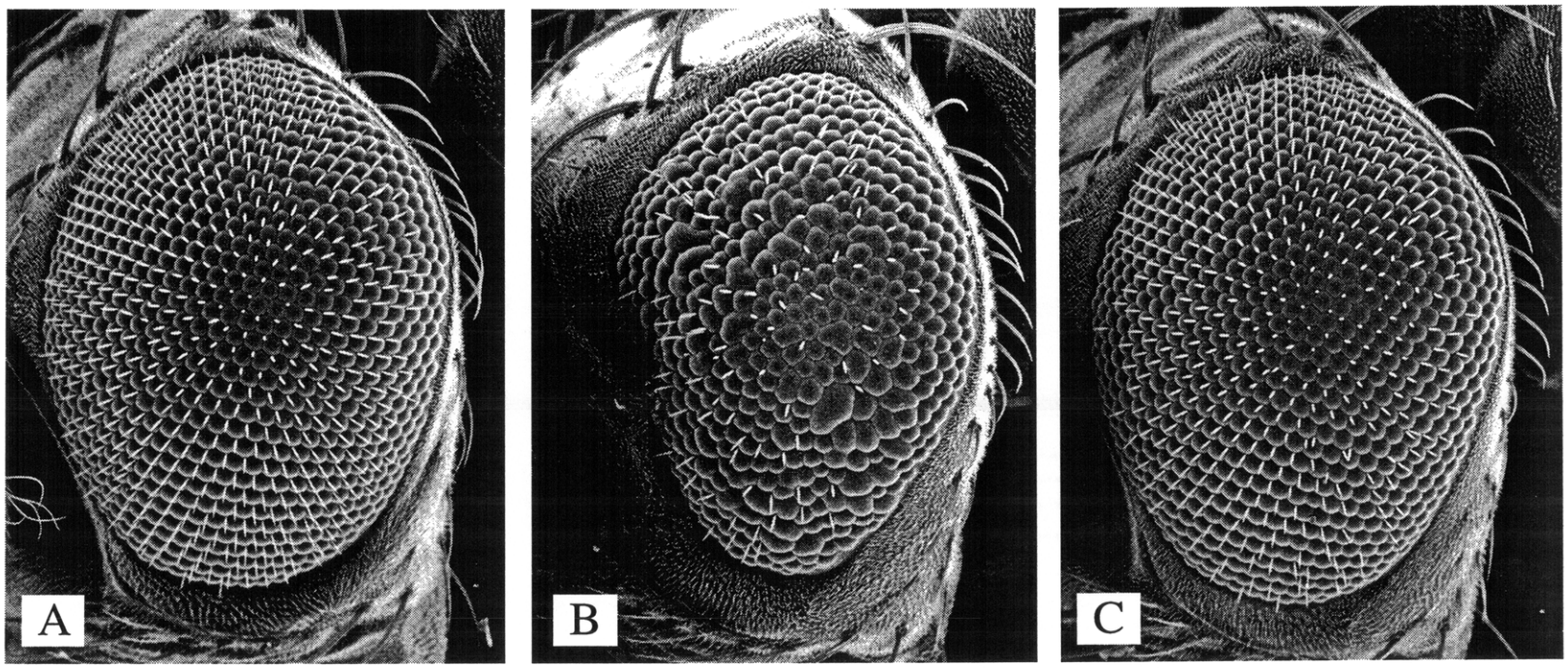


Figure 5. Overexpression of *dDP* rescues the eye phenotype of *dDP* mutants.

Shown are SEMs of Drosophila compound eyes at 200 X magnification.

The genotypes are:

(A) *Df(2R)vg-56/+*.

(B) dDP^{a2}/dDP^{a1} with a *hsdDP* transgene raised at 25°C. The reduced size of the eye and aberrant morphology are indicative of a proliferation defect in the *dDP* mutant.

(C) dDP^{a2}/dDP^{a1} with a *hsdDP* transgene raised at 25°C with daily heat shocks at 37°C. Overexpression of *dDP* completely rescues the size and morphology of the *dDP* mutant eye.

of dDP^{a1}/dDP^{a2} mutant animals. Thus, the late lethality of *dDP* mutants is not a manifestation of a defect in the early development of the organism, but rather it stems from defects in larval / pupal life.

We tested whether the lethality of *dDP* mutants was a result of disrupting the E2F/DP heterodimer by asking whether overexpression of *dE2F* could suppress the *dDP* mutant phenotype. The *dE2F* transgene under the inducible heat shock promoter was crossed into the dDP^{a1} and dDP^{a2} background. The dDP^{a1} allele was chosen because this mutation changes a single conserved residue in the E2F/DP heterdimerization domain (Girling, et al. 1993; Hao, et al. 1995). The dDP^{a2} mutation truncates the protein and is predicted to delete regions necessary for E2F binding, providing a useful comparison for the dDP^{a1} mutation. Mild overexpression of *dE2F* at 25°C resulted in significant rescue of the dDP^{a1}/Df mutant phenotype (Table 2). At 37°C the rescue conferred by the ectopic expression of *dE2F* is dampened (Table 2). This may result either from high levels of ectopic *dE2F* expression being detrimental (Asano, et al. 1996), or it may be due to fewer dDP^{a1}/Df adult escapers arising at 37°C. In contrast to the effect on dDP^{a1}, overexpression of *dE2F* provided no rescue of the dDP^{a2} mutants (data not shown). The ability of ectopic *dE2F* to rescue phenotypes in the dDP^{a1} but not the dDP^{a2} mutants suggests that despite the alteration in the DEF box of the dDP^{a1} allele, the mutant DP protein is still capable of binding E2F. This observation also indicates that the *dDP* phenotypes are a result of a failed E2F/DP transcription factor activity.

Genetic interactions between the E2F/DP heterodimer and *cyclin E*

We investigated whether *dDP* is necessary for *cyclin E* expression. In embryos that were dDP^{a1}/Df, *cyclin E* expression was not detectable in the endodomains but present in the CNS (Fig. 6A, B), a pattern comparable to that seen in homozygous $dE2F^{91}$ mutant embryos (Fig. 6C). Because the levels of *cyclin E* transcripts are so

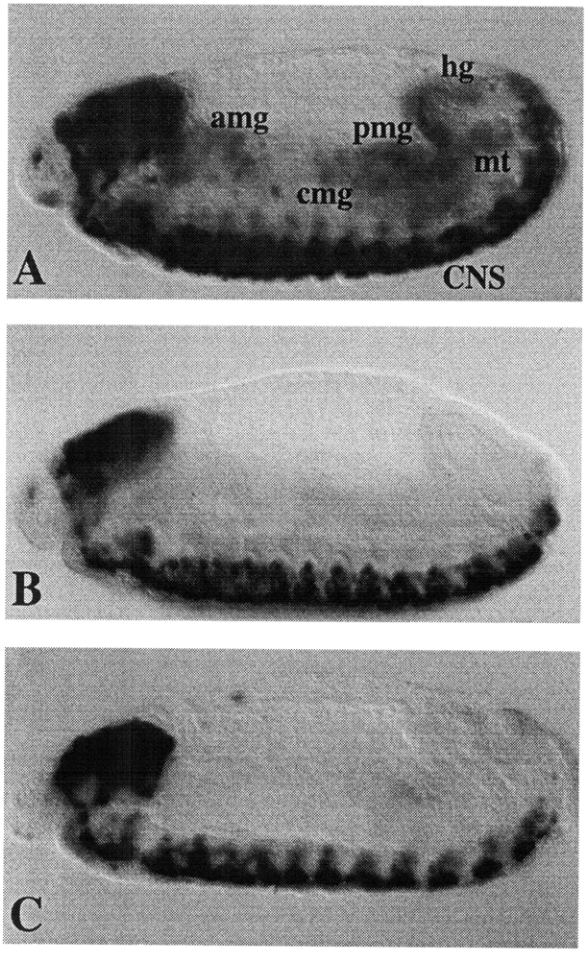


Figure 6. *cyclin E* expression in *dDP* and *dE2F* mutant embryos.

Cyclin E transcripts were detected by whole-mount in situ hybridization.

(A) In wild-type 10.5 hour embryos *cyclin E* is expressed at high levels in the mitotically proliferating CNS and at low levels in the endodomains. (The endodomains are labeled as in Fig. 1).

(B) In dDP^{a1}/Df 10.5 hour mutant embryos *cyclin E* is expressed at high levels in the CNS but is undetectable in the endodomains.

(C) $dE2F^{91}$ homozygous mutant 10.5 hour embryos show the same phenotype. *cyclin E* is expressed at high levels in the CNS but is undetectable in the endodomains.

much higher in the nervous system than in the endodomains, it is difficult to assess whether the transcript levels are reduced in the mutants in the CNS.

The *cyclin E* mutant phenotype is more severe than that of the *dE2F* and *dDP* mutants, even though *cyclin E* transcription requires the E2F/DP transcription factor. In contrast to the near normal pattern of replication seen in *dE2F* and *dDP* mutant embryos, DNA replication was not detected in the endodomains of embryos homozygous for *cyclin* E^{PZ5} (Fig. 1H). In this *cyclin E* allele S phase continued in the CNS in late embryos (Fig. 1H). No embryonic lethality resulted from the cross of *cyclin* E^{PZ5} heterozygous mothers to fathers heterozygous for a *cyclin E* deficiency, rather the *cyclin* E^{PZ5}*/Df* transheterozygotes died as early first instar larvae. Since BrdU incorporation is not seen in *cyclin E* mutant embryos in the endodomains, this shows that the embryonic endocycles are not required for hatching.

We tested whether persistent maternal levels or low levels of zygotic expression of *cyclin E* transcript accounted for the viability of *dE2F* mutant larvae. In contrast to the pupal lethality normally seen with $dE2F^{91}/dE2F^{7172}$, reducing the maternal dosage of *cyclin E* dramatically enhanced the lethality. This resulted in early larval lethality, with only 5% of the *dE2F* mutant larvae surviving until the second to small third instar larval stage. Reducing the dosage of the *cyclin E* gene in the father also enhanced the *dE2F* lethality, but to a lesser extent. In this cross 30% of the *cyclin* E^{PZ5}*/+;*$dE2F^{91}/dE2F^{7172}$ progeny survived until the second to third instar larval stage. The pronounced effect of reducing the gene dosage in mother indicates that persistent maternal pools of *cyclin E* transcript or protein permit viability of *dE2F* mutant animals. In addition, zygotically provided transcripts also contribute, but we cannot distinguish whether this is due to persistence of constitutively expressed transcripts or cyclic transcription below our detection limit.

Discussion

From a genetic screen to identify mutants that fail to undergo the S phase transcriptional program during embryogenesis we recovered five mutations in the Drosophila *DP* gene. The mutant phenotype reveals that *dDP*, like *dE2F*, is required for viability in Drosophila and that the gene has an essential role *in vivo*. The mutant phenotypes of *dDP* and *dE2F* provide insight into the functions these genes provide in controlling the cell cycle during development.

The E2F/DP transcription factor promotes S phase *in vivo*

Analysis of the activity of E2F in mammalian cell culture showed that the transcription factor was capable of driving cells into S phase when overexpressed and that this could lead to apoptosis (Heibert, et al. 1995; Johnson, et al. 1993; Lukas, et al. 1996; Shan and Lee 1994). This observation was consistent with results demonstrating that E2F could activate the transcription of a number of genes needed for the G1-S transition and the onset of S phase. However, it was also shown that when complexed with pRB the E2F transcription factor could repress the transcription of some of these genes in G1 (Weintraub, et al. 1995; Weintraub, et al. 1992; Zwicker, et al. 1996). *In trans*genic mice in which the E2F-1 gene was disrupted tumors developed (Field, et al. 1996; Yamasaki, et al. 1996). These latter two observations raised the possibility that in some tissues the E2F transcription factor could act primarily as a tumor suppressor to repress progression of the cell cycle.

The phenotypes of the Drosophila *E2F* and *DP* mutants reveal that the E2F transcription factor indeed plays a significant role as an activator of S phase *in vivo*. It was surprising that mutations in either gene have almost no effect on BrdU incorporation during embryogenesis. However, the rate of nucleotide incorporation and DNA replication appears to be slower in both mutants, and the slow larval growth rate in *dE2F* mutants is particularly striking. Because larval growth occurs

by increasing ploidy of the larval tissues via the G-S endo cycle, these phenotypes reflect a compromised ability to undergo S phase in the mutants.

In addition to the effects on polytene tissues, mitotically proliferating cells are dramatically affected by mutations in both *dE2F* and *dDP*. In the *dE2F* mutants that pupariate, either a very small adult body is formed inside the pupal case or an adult body is not formed at all. This is consistent with a defect in proliferation that reduces the number of imaginal disc cells and abdominal histoblasts that give rise to adult structures. It was previously observed that mutations in *dE2F* reduce the rate of cell growth in clones in the eye (Brook, et al. 1996). The interpretation that mutations in the *dE2F* and *dDP* genes disrupt the diploid cell cycle in the imaginal cells is supported by experiments demonstrating that overexpressing *dE2F* and *dDP* induced S phase and excess cell proliferation in the eye (Asano, et al. 1996; Du, et al. 1996b).

The defects in diploid larval cells most likely arise from aberrant cell proliferation caused by the *dE2F* and *dDP* mutations. Although we do not know the cell cycle stage affected, the demonstrated effects on polytene larval cells imply that it is the G1-S transition. Because the imaginal discs are present in *dE2F* mutants yet fail to differentiate to produce adult tissues, it is also likely that E2F has a role in post-mitotic differentiation. Such a role was previously demonstrated by clonal analysis in the eye disc (Brook, et al. 1996). A failure in differentiation of the imaginal cells could be a contributing factor in the *dDP* mutant phenotypes as well.

Although the mutant phenotypes suggest that the predominant function of the E2F transcription factor in Drosophila is to promote progression of the cell cycle, two observations raise the possibility that it may have an inhibitory role in some tissues. First, in *dDP* mutant embryos a low level of *RNR2* transcripts are present in the epidermis, and these may result from a failure to repress transcription. Second, melanotic pseudotumors develop in the *dE2F* and *dDP* mutants. We refer to these as

pseudotumors because they arise in Drosophila larvae when the lamellocytes of the immune system recognize aberrant cells, surround them, and secrete a cuticle that melanizes and becomes black (Sparrow 1978). Thus the clearest cause of melanotic tumors is an alteration of the cell surface. In the *dE2F* and *dDP* mutants this could result from perturbation of the differentiation program or apoptosis. There is evidence in Drosophila suggesting that overproliferation may contribute to melanotic tumor formation (Bryant, et al. 1993; Watson, et al. 1991; Watson, et al. 1994; Watson, et al. 1992). Although it is possible that the melanotic pseudotumors in the *dE2F* and *dDP* mutants result from hyperproliferation, we did not observe overproliferation in the larval imaginal tissues. Apoptosis may occur in some tissues in the *dE2F* and *dDP* mutants, leading to melanotic tumors. Further analysis will be required to determine whether E2F represses the cell cycle in some developmental contexts and to distinguish between these potential mechanisms for the formation of melanotic pseudotumors in the mutants.

Does *dE2F* have a function that is independent of *dDP*?

While both the *dDP* and *dE2F* mutants show late lethality, there are differences in the phenotypes resulting from mutations in the two genes. The *dE2F* mutant animals have slower larval growth than *dDP* mutants, and the *dDP* mutants develop farther as pupae. We think that the difference in strength of the phenotypes reflects the biological system rather than strength of the *dE2F* and *dDP* alleles, because we analyzed null alleles of both genes. The $dE2F^{91}$ mutation is a truncation after amino acid 31 (Duronio, et al. 1995), and dDP^{a2} is a truncation at the end of the DEF box. In mammalian cells truncation of DP after the DEF box abolishes both the E2F and DNA binding ability (Wu, et al. 1996). Given the high degree of conservation between the mammalian and Drosophila proteins, dDP^{a2} is likely to ablate DP activity.

One explanation for the less severe phenotype of the *dDP* mutations is that there is a redundant activity for *dDP* but not for *dE2F*. This could be because there are additional family members in the Drosophila genome, and these may complement the *dDP* defect better than that of the *dE2F* mutants. Alternatively, the maternal stockpiles of the *dDP* gene may persist longer than those of *dE2F*. The levels of DP protein are higher than those of E2F in the embryo (N. Dyson, personal communication).

Another explanation for the more severe effects exhibited by the *dE2F* mutants is that dE2F plays biological roles independent of dDP. Possibly the dE2F protein can act as a transcription factor as a homodimer. The rate of larval growth is influenced greatly by nutritional signals (Poodry and Woods 1990). One possibility is that dE2F links the endo and mitotic cell cycles to nutritional input, and it does so without requiring dDP function.

Significance of E2F/DP-dependent G1-S transcription

There is a clear correlation between the E2F/DP-dependent transcriptional activation of genes whose products are necessary for DNA replication and the onset of S phase. The implication was that this relationship was causal and that the cyclic transcription of these genes, some of which like cyclin E are known to be key regulatory genes, was necessary for normal S phase. The striking observation from the Drosophila *dDP* and *dE2F* mutants is that although cyclic transcription of *cyclin E*, *PCNA*, and *RNR2* is not detectable, S phase still occurs. Although we cannot exclude the possibility that cyclic transcription of these genes occurs at a low level driven by maternal pools of *dDP* and *dE2F*, the bursts of transcription that normally preceed S phase are not essential for the G1-S transition. In these mutants the cell cycle may be driven by basal levels of transcripts and post-transcriptional regulation. The maternal pools of components of the replication machinery can persist until late

in development, as evidenced by the fact that mutations in *PCNA* and *MCM2* cause late larval lethality (Henderson, et al. 1994; Treisman, et al. 1995).

The precise developmental control exercised over the cell cycle in Drosophila permits the *in vivo* role of cell cycle regulators to be evaluated. The ability to visualize the G1-S transcriptional program during embryogenesis enabled us to recover mutations in *dDP*. While the mutant phenotypes reveal that *dE2F* and *dDP* promote progression of the cell cycle, they reveal a distinction between the effect on E2F/DP-dependent G1-S transcription and the onset of S phase.

Materials and methods

Fly strains

The *cn bw sp* and *DTS 91* strains were provided by R. Lehmann. The *Ubx -lacZ CyO* balancer chromosome has been described previously (McCall, et al. 1994). The *cyclin* E^{PZ5} allele and the *cyclin E* deficiencies *Df(2L)TE116(R)GW1* and *Df(2L)TE116(R)GW3* were acquired from J. Roote.

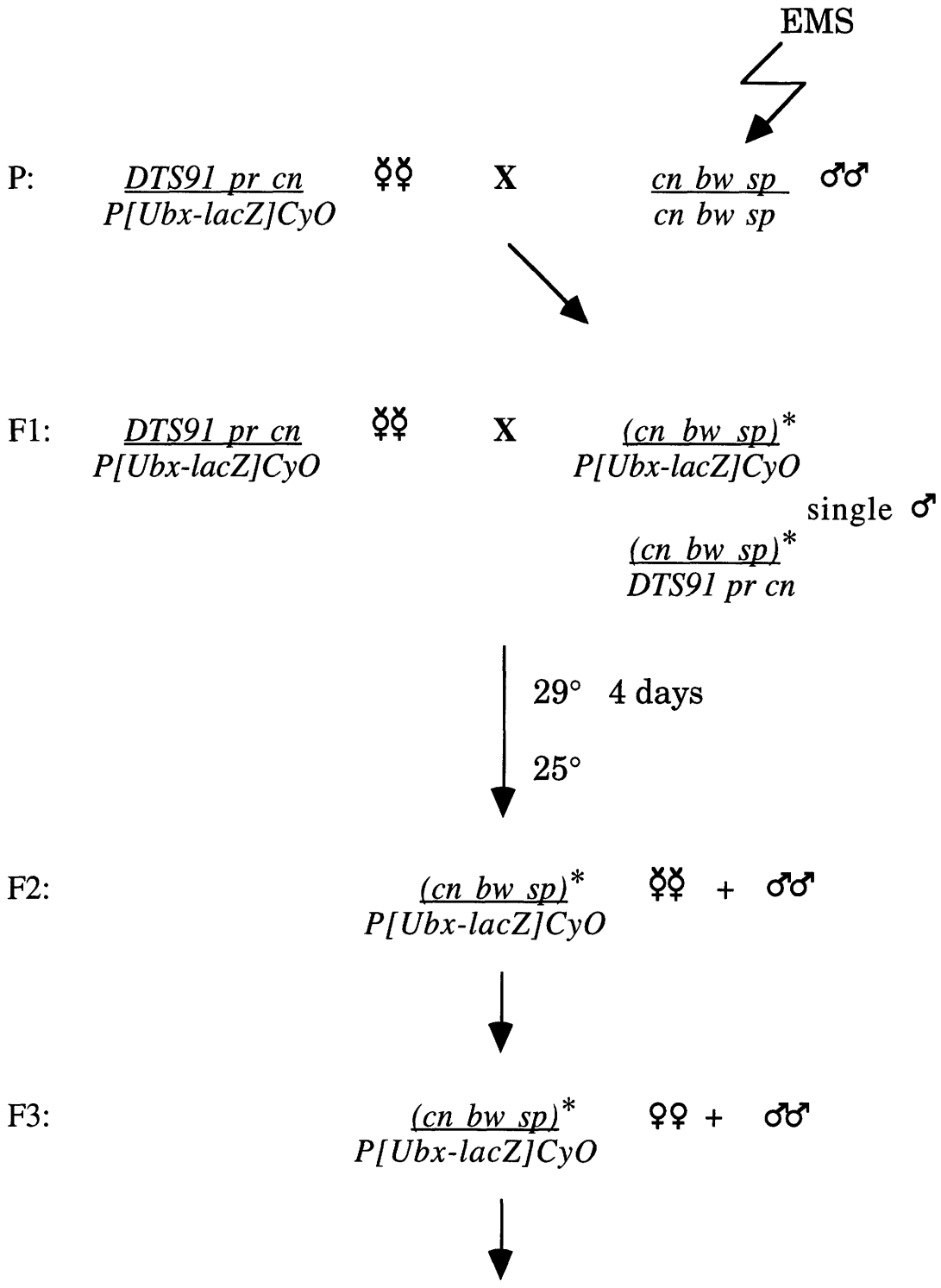
The $dE2F^{91}$ and $dE2F^{7172}$ alleles have been described previously (Duronio, et al. 1995). The *P[w+, hsp70-dE2F]*and *P[w+, hsp70-dDP]* strains were provided by N. Dyson (Duronio, et al. 1996). The deficiencies uncovering the *dDP* gene, *Df(2R)vg-33*, *Df(2R)vg-56*, and *Df(2R)vg-B*, have been described previously (Lasko and Pardue 1988; Morgan, et al. 1938; Williams and Bell 1988)and were provided by R. Duronio and the Bloomington stock center. The deficiency uncovering *dE2F, Df(3R)e-BS2* was obtained from the Bloomington stock center.

Genetic screen

Isogenized *cn bw sp* homozygous males were fed 35 mM EMS in a 1% sucrose solution for a 24 hour period (Fig. 7). Immediately after feeding, these males were crossed to virgin females heterozygous for a chromosome carrying a dominant temperature-sensitive mutation, *DTS91 pr cn* and a *CyO* balancer carrying a *lac Z* reporter gene under the control of the *Ubx* promoter, (*Ubx-lacZ CyO*). Single male progeny from this cross with the genotypes *cn bw sp/P[Ubx-lacZ] CyO* and *cn bw sp/DTS91 pr cn* were collected and mated individually to *DTS91/P[Ubx-lacZ] CyO* virgin females. To facilitate collection of the desired *cn bw sp /P[Ubx-lacZ] CyO* virgin females and male progeny, the vials were placed at the restrictive temperature for the *DTS91* mutation, 29°C, for three days. The adults were removed, and the vials were incubated at 29°C for an additional day before being moved to 25°C. *cn bw sp/P[Ubx-lacZ] CyO* virgin female and male progeny from this cross were collected

Figure 7. Screen for mutations that disrupt G1-S transcription.

Isogenized *cn bw sp* flies were mutagenized with EMS. The *DTS91* chromosome was used to select for flies bearing the mutagenized chromosome over a balancer expressing *lacZ*. From the F3 adults, 8-15 hour embryos were collected and hybridized in situ with *PCNA* and *lacZ* riboprobes. Embryos that did not express *lacZ* were homozygous for the mutagenized second chromosome. F3 lines that gave rise to progeny with aberrant *PCNA* expression were maintained.



Collect embryos and examine by in situ hybridization.

and mated to establish mutant lines. 8-15 hour embryos were collected from these lines and hybridized *in situ* with a *PCNA* riboprobe. To distinguish between embryos homozygous for the *cn bw sp* mutagenized chromosome and their heterozygous *cn bw sp/Ubx-lacZ* siblings, the embryos were simultaneously hybridized with a *lacZ* riboprobe.

In situ hybridization

In situ hybridization was carried out essentially as described previously, (Tautz and Pfeifle 1989). To perform *in situ* hybridization on large numbers of independent samples multiwell baskets were used. Hybridization was carried out at 65°C. Digoxygenin labelled antisense RNA probes were made as described in the Boehringer Mannheim kit. The *RNR2* clone used to make probe was obtained by PCR amplification of Oregon R genomic DNA using degenerate primers (Duronio and O'Farrell 1994). The PCR products were cloned into Bluescript KS. *Cyclin E* probes were made from the E41-1 clone obtained from H. Richardson (Richardson, et al. 1993). The *PCNA* probe was made from a full length cDNA isolated in this laboratory by J. M. Axton from the library generated by N. Brown (Brown and Kafatos 1988). *lacZ* probes were made from the pC4βgal plasmid (Thummel, et al. 1988).

Sequence analysis of the *dDP* alleles

Transheterozygous *dDP/Df(2R)vg-B* third instar larvae were collected from a cross of *dDP/T(2;3)TSTL*^{14} and *Df(2R)vg-B/T(2;3)TSTL*^{14} adults by selecting non-Tb larvae. *T(2;3)TSTL*^{14} is a translocation between the balancers *SM5* and *TM6B* that carries the dominant larval / pupal marker *Tubby (Tb)* (Gatti and Goldberg 1991). To recognize polymorphisms between our strains and the published sequence, we isolated DNA from adults that were transheterozygous for two unrelated lines created in our screen. Genomic DNA was isolated from these larvae and adults and a

1400 base pair region encompassing approximately amino acid 15 to 377 (Dynlacht, et al. 1994) was amplified by the polymerase chain reaction. Because two different 5' ends have been described for the *dDP* gene (Dynlacht, et al. 1994; Hao, et al. 1995), the amplified region contained the conserved domains but not the 5' end of the gene. The amino acid numbering is that of Dynlacht et al. (1994). Primer sequences were '5 CTTTAGTCAGATGGGCAGTCAAG3' and 5'CTGTAACTAACTCGACTACCAC3'. 10-15 separate PCR reactions were pooled and sequenced directly in the Whitehead sequencing facility using fluorescence automated sequencing. Primers for sequencing were spaced at approximately 300 base pair intervals.

BrdU labeling

8 - 15 hour embryos were labeled with BrdU according to a protocol obtained from Rolf Bodmer, a modified version of Bodmer, et al. (1989). After permeabilization, the embryos were incubated with BrdU for either 10 or 40 minutes at room temperature. The fixed embryos were hydrolyzed in 2N HCl for 70 minutes. The 70 minute acid treatment is the key difference from the published protocol and provides better detection of the label. The balancers used to maintain the *dDP* and *dE2F* mutant lines were marked with a *Ubx-lacZ* transgene. An anti-β-galactosidase antibody (Promega) was used in conjunction with the anti-BrdU antibody to distinguish the 25% homozygous mutant embryos from their siblings. The antibody staining was detected using the horseradish peroxidase histochemical reaction (Bodmer, et al. 1989).

Lethal phase

Embryonic lethality was assessed by mating *dDP allele* / + females to *Df(2R)vg-B* / *CyO* males. The parents were allowed to mate at 25°C for several days to reduce the number of unfertilized eggs in the collections. The eggs were collected

on apple juice plates at 25°C and counted. The number of eggs that hatched was recorded 24 hours later. The same procedure was carried out for alleles of *dE2F*. The furthest developmental stage reached by the *dDP* alleles *in trans* to each other or *in trans* to *Df(2R)vg-B* was determined by generating stocks with the *dDP* alleles and the deficiency over $T(2;3)TSTL^{14}$ (Gatti and Goldberg 1991). The *dE2F* alleles were balanced over *TM6B* to determine the lethal phase.

To test for the effect of reducing the dosage of *cyclin E* on the viability of *dE2F* mutants two reciprocal crosses were done. The effect of zygotic expression alone was examined by crossing $dE2F^{7172}$ / *TM6B* females to $cycE^{PZ5}$; $dE2F^{91}$ / *TSTL* males. The effect of the maternal plus the zygotic contribution was tested by the reciprocal cross.

Heat shock rescue experiments

w; Df(2R)vg-56 / CyO females were mated to *w;* dDP^{a1} */ CyO ;* $P[w^{+};hs\text{-}dDP]$/+ or *w;* dDP^{a1}*/CyO ;* $P[w^{+};hs\text{-}dE2F]$/+ males at 25°C (Duronio, et al. 1996). All the progeny from the cross were counted and placed into genotypic classes. To test for rescue of dDP^{a1}/dDP^{a2} transheterozygotes, dDP^{a2}*/CyO* females were crossed to *w;* dDP^{a1}*/CyO;* $P[w^{+};hs\text{-}dDP]$*/TM3, Sb* males at 25°C. No dDP^{a1}/dDP^{a2} adults were observed.

For the heat shock treatments, embryos were collected (from the crosses shown above) for 24 hours at 25°C in vials. The heat shock treament was administered by placing the vials in a 37°C air incubator for an hour and then the vials were returned to 25°C. The heat shocks were initiated either immediately after the completion of a 24 hour collection or after the eggs were allowed to develop at 25°C for 5-6 days. In either case, subsequent heat shocks were delivered two times daily.

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Chapter Four

E2F/DP controls the activity of DNA replication origins

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Abstract

We demonstrate that in Drosophila E2F/DP controls differential regulation of replication origins within S phase. In the nurse cells of the ovary, E2F/DP limits the use of replication origins in the heterochromatin. In follicle cells, E2F/DP plays a dual role in the transition from genomic replication to the amplification of chorion loci; it is required to shut off genomic replication and to activate amplification. In a *dE2F* mutant that lacks the C-terminal transcriptional activation and RB-binding domain we observed over-amplification. In addition to the positive and negative effects on replication origins, we show that E2F/DP is required for the transport of nurse cell contents to the developing oocyte and subsequent nurse cell apoptosis.

Introduction

A significant body of work suggests that the E2F transcription factor plays a pivotal role in the control of S phase entry. The transcription factor is a heterodimer of E2F and DP proteins, and there are several forms of both proteins in mammalian cells (Weinberg, 1995). E2F-binding sites are found in the promoters of genes that are required for DNA synthesis and those that regulate S phase entry (Nevins, 1992). E2F sites can act both as positive and negative elements depending on whether E2F is free or complexed with a pocket protein such as the retinoblastoma protein (RB) (Weintraub et al., 1992). The E2F/DP heterodimer tethers RB to E2F-dependent promoters through a small RB-binding region embedded in the transactivation domain of the E2F protein (Helin et al., 1993). In addition to occluding the E2F activation domain, RB actively represses transcription at these promoters and can affect the state of chromatin condensation through an interaction with a histone deacetylase (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Weintraub et al., 1995). Late in G1, phosphorylation of RB by cyclin D/CDK4,6 frees E2F/DP and triggers the expression of E2F-dependent genes including cyclin E (Botz et al., 1996; Ewen et al., 1993; Geng et al., 1996; Kato et al., 1993; Ohtani et al., 1995). The cyclin E/CDK2 complex also phosphorylates RB and creates a positive feedback loop that is thought to result in an irrevocable commitment to S phase (Sherr, 1996).

It is not clear whether the predominant role of E2F *in vivo* is negative or positive. Ectopic expression of the best characterized E2F protein, E2F-1, is sufficient to drive serum-starved, quiescent fibroblasts through S phase, suggesting a positive role in S-phase entry. (Johnson et al., 1993; Qin et al., 1994). The E2F-1 induced fibroblasts then become apoptotic. The induction of apoptosis, however, may be a secondary consequence of driving cells into an ectopic S phase. Recent studies indicate that the induction of DNA synthesis and apoptosis are separable functions of

E2F-1. Ectopic expression of a transactivation defective *E2F-1* mutant induces cells to undergo apoptosis but not DNA replication (Hsieh et al., 1997; Phillips et al., 1997). The importance of the transcriptional activation domain of E2F-1 for the induction of S phase was established in a previous study (Qin et al., 1995). Furthermore, overexpression of a dominant-negative *DP-1* mutant results in a block to G1-S progression (Wu et al., 1996). These findings suggest that E2F-1 may be a transcriptional activator of G1-S progression and a repressor of apoptosis.

Analyses of mice lacking a functional *E2F-1* gene have challenged the models established by the overexpression studies. *E2F-1*$^{-/-}$ mice are viable and fertile but develop tumors in a variety of tissues (Field et al., 1996; Yamasaki et al., 1996). In addition, large and sometimes binucleate cells are detected in the exocrine gland, and *in vitro* cultured *E2F-1*$^{-/-}$ thymocytes are less susceptible to apoptosis than their wild-type counterparts. The predisposition to tumors and exocrine gland dysplasia suggest a previously unrecognized tumor suppressor function for E2F-1. Moreover, these studies indicate that E2F-1 is either not critical for entry into S phase or that other members of the E2F family (E2F-2 through -5) can compensate for this function. It is not clear whether the overexpression studies or the knockout mice come closer to reflecting the physiological role of E2F-1. Isolation of a range of mutations in addition to the null would provide significant insights into the function of E2F-1 *in vivo*.

Drosophila homologs to E2F, DP, and an RB-like protein (RBF) have been identified (Du et al., 1996a; Dynlacht et al., 1994; Hao et al., 1995; Ohtani and Nevins, 1994). As in mammalian cells, ectopic *dE2F* expression is sufficient to drive quiescent cells into S phase and apoptosis (Asano et al., 1996; Du et al., 1996b).We have shown that null mutations in *dE2F* and *dDP* cause lethality late in development with some tissues being underdeveloped or absent (Royzman et al., 1997). A positive role for the E2F/DP heterodimer in cell cycle progression was inferred from these

defects. In addition, G1-S transcription of *cyclin E* and genes encoding replication functions (*proliferating cell nuclear antigen* and *ribonucleotide reductase 2*), normally observed in late embryogenesis, is missing in the *dE2F* and *dDP* mutants (Duronio et al., 1998; Duronio et al., 1995; Royzman et al., 1997). Despite the pronounced effect on E2F-dependent G1-S transcription, a block to replication was not observed. However, replication did appear slower.

Drosophila oogenesis makes it possible to examine aspects of DNA replication that are not readily apparent during embryogenesis (for review see Spradling and Orr-Weaver, 1987; Spradling, 1993). Ovarian nurse cells and follicle cells undergo a set of mitotic divisions before switching to an endo cycle (a cycle consisting of only S phase and a gap phase) and becoming polyploid. During polyploidization, the parameters of S phase are altered such that differential regulation of replication origins throughout the genome occurs. In both the nurse cells and follicle cells, the heterochromatin is underreplicated. In the follicle cells a few genomic intervals escape restrictions on re-replication, and these regions continue to be amplified after the cessation of genomic replication. Weak alleles of essential cell cycle genes exhibit defects in oogenesis for two reasons. Polyploidization and amplification place demands on DNA replication factors. In addition, maternal pools of most cell cycle regulators and components of the DNA replication machinery are depleted in the adult. Thus, weak alleles of *cyclin E, PCNA,* and *orc2* are female sterile (Henderson et al., 1994; Landis et al., 1997; Lilly and Spradling, 1996).

Here we report the identification and analysis of new mutations in Drosophila *dE2F* that cause cell cycle defects in oogenesis. These mutations, in addition to a previously isolated female-sterile allele of *dDP* (Royzman et al., 1997), allowed us to define the role of E2F in DNA replication. We demonstrate that in different developmental contexts E2F/DP is needed to inactivate or activate DNA replication

origins. In addition, we show that the E2F/DP heterodimer is required for nuclear lamin breakdown and developmentally-induced apoptosis *in vivo*.

Results

Female-sterile mutations in the *dE2F* gene

We recovered two viable mutations in the gene for the *dE2F* subunit of the E2F transcription factor from a screen for mutations affecting G1-S transcription during embryogenesis. The *PCNA* gene, a target of E2F, is transcribed in a stereotypic pattern that anticipates DNA replication late in Drosophila embryogenesis (Duronio and O'Farrell, 1994). This pattern was used as the basis for a screen to identify regulators of the G1-S transition, as previously described (Royzman et al., 1997). From 1000 mutagenized third chromosome lines established by Moore et al. (1998), we focused on two mutants with reduced *PCNA* expression during embryogenesis (data not shown).

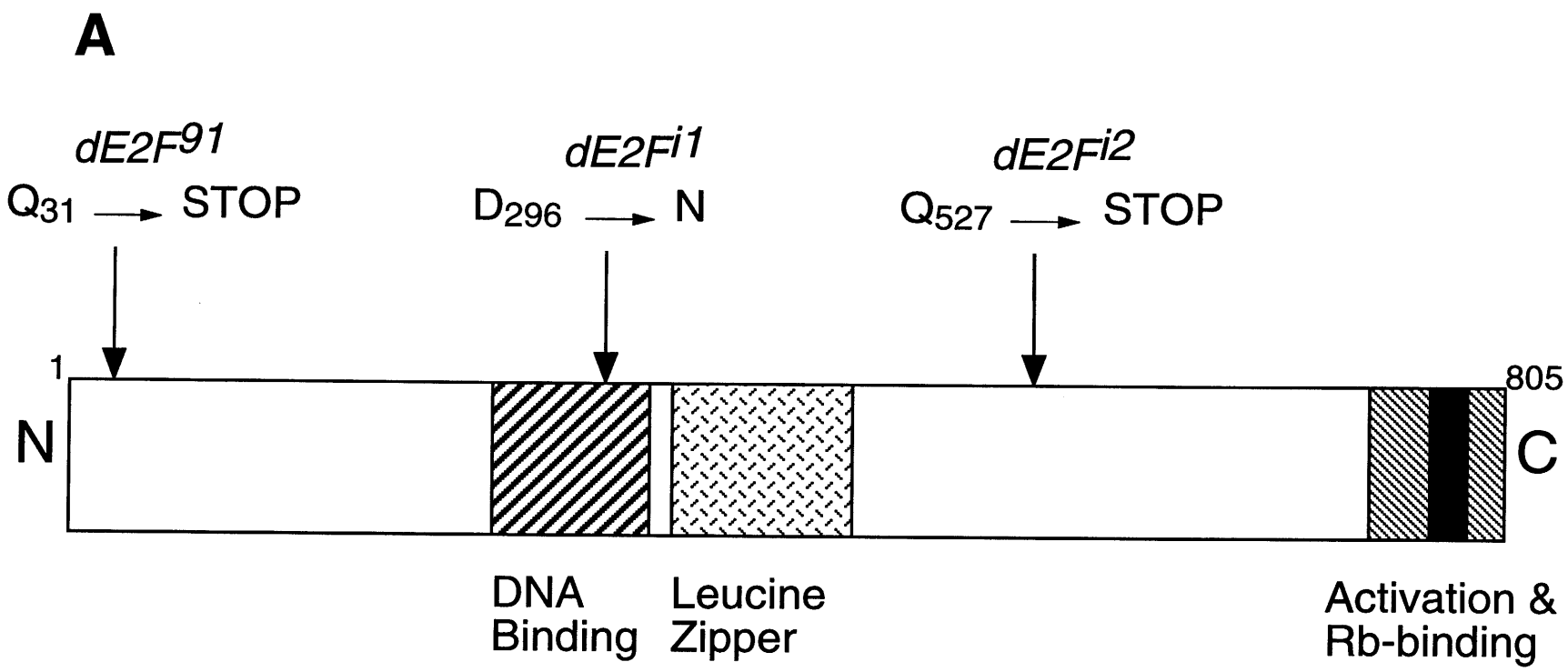
We sought to determine if the two mutations were alleles of the *dE2F* gene. One mutation when placed *in trans* to a deficiency or a null *dE2F* allele caused reduced viability. The recovered adults were female sterile and had eye and bristle defects. The mutant females were able to lay eggs, but the eggs failed to develop. These phenotypes closely resemble those observed for a weak allele of *dDP*, dDP^{a1} (Royzman et al., 1997). The other mutation was fully viable *in trans* to a deficiency or null *dE2F* allele; these adults had slightly rough eyes and the females had reduced fertility. Unexpectedly, the two alleles were viable and fertile *in trans* to each other. Thus, the complementation data were ambiguous and left open the possibility that these were either alleles of *dE2F* or mutations in interacting genes.

To determine if the mutations were in the *dE2F* gene, we sequenced the *dE2F* coding regions from the mutants. Both mutants had changes in the *dE2F* open reading frame (Fig. 1). The mutation that is semi-lethal *in trans* to a deficiency is a G to A nucleotide transition that converts amino acid Asp^{296} of the *dE2F* DNA-binding domain to Asn. This residue lies in a region that is highly similar between the E2F and DP families of proteins, and it is conserved in all known Drosophila and

Figure 1. Position and nature of new *dE2F* alleles.

(A) A schematic of the dE2F protein with the conserved regions indicated: the DNA-binding domain, leucine zipper, transcriptional activation domain, and the embedded RB-binding region in black. The positions and the amino acid changes in $dE2F^{i1}$ and $dE2F^{i2}$ are shown relative to $dE2F^{91}$, the previously characterized null allele (Duronio et al., 1995; Royzman et al., 1997).

(B) The *dE2F* DNA-binding domain includes a region of considerable similarity between the DP and E2F families of proteins. This block of amino acids is called the DEF box in the DP gene, and it is important for E2F/DP dimerization and the recognition of E2F/DP binding sites in the promoters of E2F/DP regulated genes (Bandara et al., 1993; Hao et al., 1995). The DEF consensus for Drosophila DP and E2F proteins is shown (modified from Bandara et al., 1997). A broader consensus to include mammalian DPs (1-3) and E2Fs (1-5) as well as the recently identified E2F-like protein, E2F6/EMA (Morkel et al., 1997; Trimarchi et al., 1998) is indicated in bold. The positions of mutations dDP^{a1}, $dE2F^{i1}$ and dDP^{a2} are shown within the DEF consensus. dDP^{a1} is an amino acid substitution, while dDP^{a2} is a stop codon. Note that the affected residues are perfectly conserved in all known Drosophila and vertebrate E2F and DP families of proteins.



B

DEF Consensus **R R -Y D - - N V** L - - - N - I - **K - - K** - - I - **W**

dDPa1 *dE2Fi1* *dDPa2*

vertebrate E2F and DP proteins (Fig. 1B) (Bandara et al., 1997; Hao et al., 1995). The mutation was named $dE2F^{i1}$. The finding that the $dE2F^{i1}$ mutation is analogous to the missense change in the DNA-binding region in the dDP^{a1} allele may explain the similarity of the phenotypes exhibited by the two mutants (see Fig. 1B). The second mutation, $dE2F^{i2}$, is a C to T change that converts Gln^{527} to a stop codon, resulting in the deletion of the transcriptional activation and RB binding domains. The C-terminal domain of the Drosophila E2F protein shown to be both necessary and sufficient for transcriptional activation (Ohtani and Nevins, 1994) is completely missing in this mutant.

Phenotypic analysis of Drosophila $E2F^{91}$, a null allele that lacks all the recognized functional domains of the E2F family of proteins, established that *dE2F* is essential for viability and normal growth (Duronio et al., 1995; Royzman et al., 1997). However, viability and growth are not affected in the $dE2F^{i2}$ mutant. Thus, the $dE2F^{i2}$ mutant appears to retain significant physiological function. This was also evidenced by another observation. Cyclin E is a critical target of E2F in Drosophila (Duronio et al., 1996). We tested whether reducing the dosage of the *cyclin E* gene by half would effect the viability of $dE2F^{i2}/dE2F^{91}$ flies and found that it had no effect. In contrast, the same reduction enhanced the lethality of the $dE2F^{i1}/dE2F^{91}$ mutant organisms. We were not able to recover $dE2F^{i1}/dE2F^{91}$ adults that were heterozygous for a strong but completely recessive *cyclin E* mutation.

dE2F and *dDP* limit replication of heterochromatin

The female sterility of the dDP^{a1}, $dE2F^{i1}$, and $dE2F^{i2}$ mutants suggested there were defects in oogenesis. Although the ovaries were small, the cell cycle appeared normal in the mutants. The mutant egg chambers contained 16 germline cells, 15 nurse cells and an oocyte, indicating the proper completion of the four mitotic

divisions that produce the egg chamber cyst. In addition, the nurse and follicle cells were undergoing polyploidization as evidenced by the size of the nuclei.

Although the nurse cells were polyploid, the properties of endoreplication were affected in all three mutants. Normally, replication in nurse cells is incomplete, with sequences residing in heterochromatic regions of the genome being underrepresented (Fig. 2A,B) (Hammond and Laird, 1985; Lilly and Spradling, 1996). Staining with the DNA dye DAPI gives one or a few small, intensely labeled, chromocenters, aggregates of heterochromatic sequences, in each nurse cell nucleus. It has been shown that size of the chromocenter reflects the levels of heterochromatic sequences, and mutations that restore late replication to polyploid cells result in a much larger chromocenter (Lilly and Spradling, 1996). DAPI staining of $dE2F^{i2}/Df$ mutant ovaries showed a dramatic increase in the size of the nurse cell chromocenter (Fig. 2C), whereas underreplication was normal in the heterozygous controls (Fig. 2A). The same relative over-replication of heterochromatic regions was observed for $dE2F^{i1}$ and dDP^{a1} (data not shown).

The mutant phenotype suggests that E2F/DP influences the use of late replicating origins. To establish that this effect was due to loss of E2F function and not a novel E2F activity in these mutants, we rescued the lethality of null $dE2F^{91}$ and dDP^{a2} mutants by their respective *hsp70-dE2F* and *hsp70-dDP* transgenes. A partial rescue of lethality was achieved with basal expression of the wild-type genes from the *hsp70* promoter. These flies would be expected to have reduced E2F activity relative to wild-type, however, they retain enough function to be viable. The recovered *hs-dE2F/+;* $dE2F^{91}/Df$ and dDP^{a2}/Df; *hs-dDP/+* mutant adults had rough eyes and were female sterile. DAPI staining revealed that the nurse cell chromocenters in the mutants were increased in size and intensity as compared to controls (data not shown). These results establish that the increased presence of nurse cell heterochromatin is due to loss of E2F function.

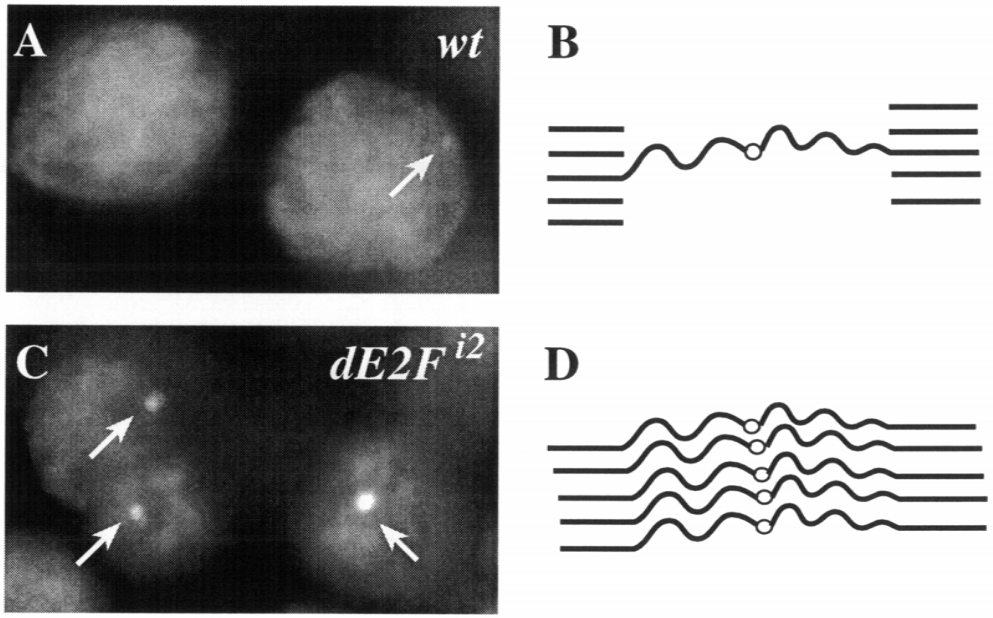
Figure 2. E2F/DP is required in nurse cells to limit the usage of late replicating origins. DAPI was used to monitor the degree of underrepresentation of heterochromatic sequences.

(A) In control $dE2F^{i2}$/+ stage 10 nurse cells underreplication was normal and the chromocenter is scarcely visible (arrow).

(B) Schematic of replication in polyploid cells: sequences residing in heterochromatic regions (curved lines) of the genome are not completely duplicated.

(C) In mutant $dE2F^{i2}/Df$ stage 10 nurse cells normally underrepresented heterochromatic sequences are restored to nurse cell nuclei. Note the large and intensely staining chromocenter in each nurse cell nucleus (arrows). The same phenotype was observed for the other female-sterile mutants, dDP^{a1}/Df and $dE2F^{i1}/dE2F^{91}$, as well as the nulls that were rescued to adulthood, dDP^{a2}/Df and $dE2F^{91}/Df$ (data not shown).

(D) Schematic showing the restoration of heterochromatic sequences in the mutants.


A
wt
B
C
dE2F i2
D

The role of *dE2F* and *dDP* in chorion gene amplification

It was of interest to determine if E2F/DP regulates other examples of differential replication. In follicle cells chorion gene clusters escape restrictions on rereplication, and amplification of these regions continues after the cessation of general genomic replication (Calvi et al., 1998). The amplification of chorion genes in follicle cells is required for the rapid synthesis of the eggshell (chorion), thus mutations that disrupt this process cause female sterility and thin eggshells. Consistent with a defect in chorion amplification, eggs laid by dDP^{a1}/Df and dDP^{a2}/Df; *hsDP*/+ mutant mothers, had abnormally thin eggshells (data not shown). The eggshells produced by the *dE2F* mutants were also indicative of an amplification defect, but the phenotype was more variable (data not shown).

To determine the role of E2F/DP in chorion gene amplification directly, we analyzed BrdU incorporation in whole-mount ovaries (Figs. 3, 4 and Table 1). In wild-type ovaries the shift from general genomic replication to amplification of chorion genes is readily observed. During genomic replication, BrdU is incorporated throughout the entire follicle cell nucleus, and groups of follicle cells in the same egg chamber undergo BrdU incorporation at different times to give an asynchronous pattern (Fig. 3A,A'). The cessation of genomic BrdU incorporation is followed by continuous amplification of chorion genes. During amplification (stages 10B-13), BrdU is incorporated in all the follicle cells at the same time but only at four subnuclear foci (Fig. 3B,B',see circle in B'). The subnuclear BrdU dots correspond to amplifying regions of the genome (Calvi et al., 1998). In the *dDP* and *dE2F* mutant ovaries, replication prior to stage 10B was normal; both labeled (S phase) and unlabeled (G phase) follicle cell nuclei were observed (data not shown). However, the mutants deviate from wild type and each other during amplification (stage 10B and later) (Table 1).

Figure 3. *dDP* is necessary for amplification and the shut off of genomic replication. (A-D) DNA replication was assayed by BrdU labeling followed by antibody staining detected by the horseradish peroxidase (HRP) reaction. BrdU incorporation is indicated by the red staining. A DAPI counterstain to detect all nuclei was also used. Panels A-D are at the same magnification, and are shown below (A'-D') at a higher magnification.

(A, A') In stage 9 control dDP^{a1}/+ egg chambers genomic replication is asynchronous among follicle cell nuclei. The egg chamber contains follicle cells that are labeled (S phase) with BrdU and those that are not labeled (G phase). The same pattern of nuclear BrdU incorporation is observed in the female-sterile *dE2F* and *dDP* mutants.

(B, B') In stage 10B control egg chambers, polyploidization of the follicle cell genome is no longer observed. Instead, synchronous subnuclear BrdU incorporation is seen. The subnuclear dots correspond to amplifying chorion clusters. The subnuclear foci from a single follicle cell nucleus are indicated by a circle. The different intensity and size of foci (one large, one medium, and two small) reflect the extent of gene amplification.

(C, C') A stage 10B dDP^{a2}/Df mutant egg chamber is shown. Genomic replication is persistent and synchronous in this mutant egg chamber. The same phenotypes were observed for the dDP^{a1}/Df mutant. Note that the nuclei are much larger than at stage 9. It is the shut off rather than the onset of polyploidization that is defective in the mutants.

(D) A stage 10B dDP^{a1}/Df egg chamber is shown. Genomic replication has been shut off but amplification is not observed. The same replication defect was observed for dDP^{a2}/Df.

(D') A magnified DAPI image of D showing that the follicle cell nuclei are in fact present and polyploid.

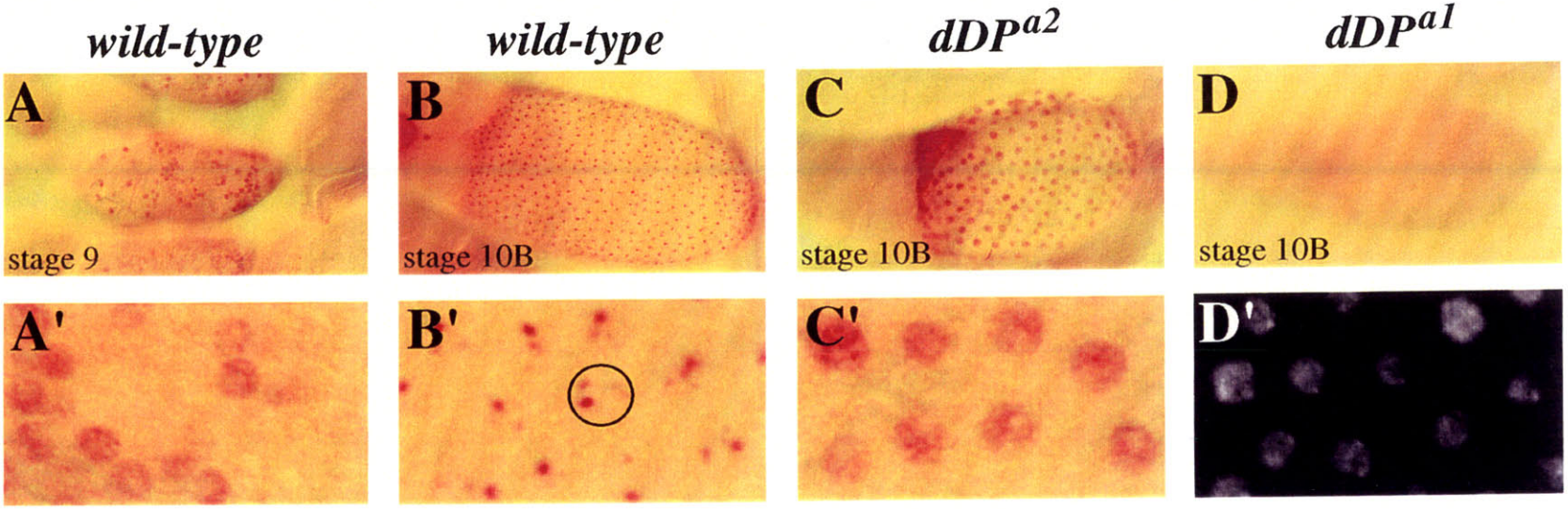

wild-type
wild-type
dDP^a2
dDP^a1
A
stage 9
B
stage 10B
C
stage 10B
D
stage 10B
A'
B'
C'
D'

Table 1. Replication phenotypes of *dE2F* and *dDP* mutants in follicle cells

Genotypes	Genomic Replication[c] (Fig. 3C)	Chorion Amplification				Total # of 10B-11 Egg Chambers
		Absent (Fig. 3D)	Reduced (Fig. 4A)	Normal (Fig. 3B)	Increased (Fig. 4C)	
dDP^{a1}/+	0%	16%	8%	76%	0%	38
dDP^{a1}/*Df*	18%[d]	82%	0%	0%	0%	51
$dE2F^{il}$ / $dE2F^{91}$ or $dE2F^{7172}$[a]	5%[e]	43%	30%	21%	0%	61
$dE2F^{i2}$ / $dE2F^{91}$ or *Df*[b]	0%	10%	11%	19%	60%	42

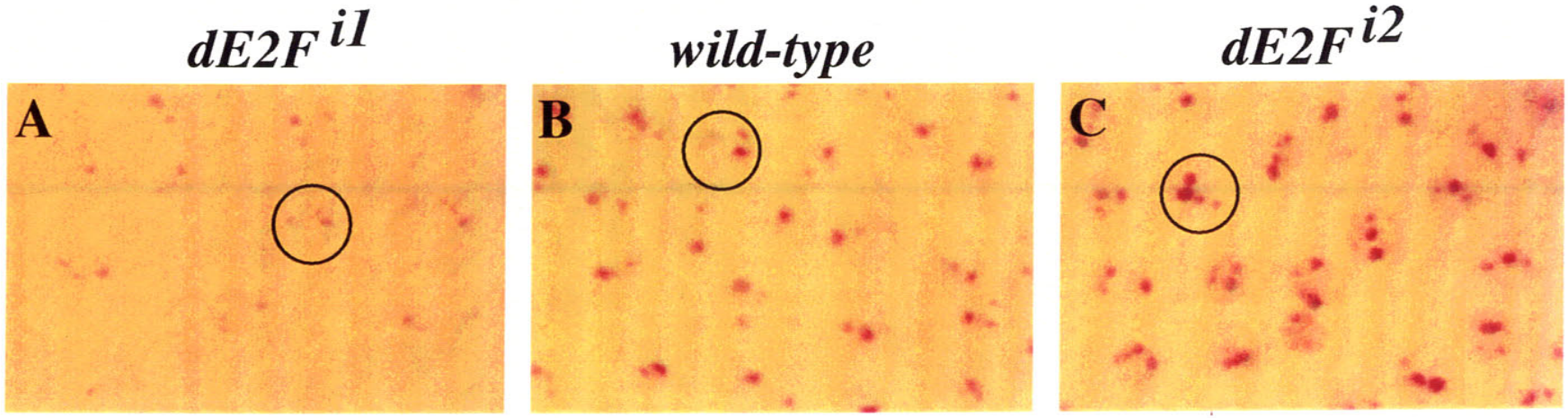
[a] The data are combined for genotypes $dE2F^{il}/dE2F^{91}$ and $dE2F^{il}/dE2F^{7172}$.
[b] The data are combined for genotypes $dE2F^{i2}/dE2F^{91}$ and $dE2F^{i2}/Df$.
[c] Genomic follicle cell replication is inappropriate at stages 10B - 11.
[d] Genomic replication occurred in synchrony in all the follicle cells.
[e] Genomic replication persisted in a few follicle cells.

BrdU labeling of the dDP^{a1}/Df and dDP^{a2}/Df; *hsDP*/+ mutants along with a DAPI counterstain revealed that the follicle cell nuclei were present but failed to initiate amplification (Fig. 3D,D'). In addition, genomic replication continued in some of these egg chambers (Fig. 3C,C' and Table 1). It is striking that this round of genomic replication is synchronous, a property of normal amplification (compare Fig. 3C' to 3A' and 3B'). Thus it cannot be the consequence of follicle cell genomic replication being delayed relative to egg chamber development. The difference between the phenotypes shown in Fig. 3C and Fig. 3D is likely to result from variation in the levels of active dDP among egg chambers. Taken together, the phenotypes suggest that *dDP* plays a dual role in the regulation of replication in follicle cells. It is required to shut off genomic follicle cell replication at the same time that it is needed to activate chorion gene amplification. The results of the BrdU analysis directly correlate with the severe eggshell defects observed for the *dDP* mutants.

BrdU labeling of $dE2F^{i1}/Df$ mutant egg chambers showed that *dE2F* also is required for amplification; subnuclear BrdU incorporation was either absent or greatly reduced in these mutant egg chambers (compare Fig. 4A to 4B). The $dE2F^{i2}$ mutation, however, had the opposite effect on amplification. BrdU labeling of $dE2F^{i2}$ mutant ovaries revealed significantly larger subnuclear dots (stage 10B and in the later stages) than those observed in wild type (compare Fig. 4C to 4B). In addition to the over-amplifying follicle cells, some egg chambers had small patches that completely failed to incorporate BrdU (data not shown). Thus it appears that in the presence of truncated E2F, the majority of follicle cells initiate amplification, but cannot limit the rounds of reinitiation of DNA synthesis within each chorion gene cluster. The $dE2F^{i2}$ mutant phenotype also shows that C-terminal transcriptional activation and RB-binding domains of *dE2F* are not required for either repression of genomic replication or the onset of amplification.

Figure 4. *dE2F* is necessary for normal amplification.

DNA replication in stage 10B egg chambers was assayed by incorporation of BrdU (red staining). Panels A-C are at the same magnification. In $dE2F^{i1}/Df$ mutant egg chambers (A) amplifying chorion loci are reduced in size and intensity as compared to wild-type (B). In the wild-type egg chamber most follicle cell nuclei have 4 spots of different intensities (circle). It is sometimes difficult to see them in the same focal plane. In $dE2F^{i2}/Df$ mutant egg chambers (C) the size and intensity of the amplifying chorion loci is greatly increased. The mutant phenotype makes it easier to see the four subnuclear dots within each follicle cell nucleus (circle).


dE2F i1
wild-type
dE2F i2
A
B
C

Expression of *dE2F* correlates with the requirement for E2F/DP activity

Because E2F/DP is required for oogenesis, we examined the expression of *dE2F* and *dDP* in wild-type ovaries. The *dDP* transcript was constitutively high at all stages of oogenesis (data not shown). To examine *dE2F* expression, we used a *dE2F* enhancer trap line, $dE2F^{rm729}/+$ (Brook et al., 1996; Duronio et al., 1995). β-galactosidase staining of ovaries from $dE2F^{rm729}/+$ females revealed a striking developmental pattern. *dE2F* transcript was observed at high levels in follicle and nurse cell nuclei starting at stage 10B (Fig. 5A). The induction of *dE2F* correlates with the completion of genomic polyploidization and the transition to amplification in follicle cells. To analyze the expression of dE2F protein in wild-type and mutant ovaries, we used antibodies against dE2F (Asano et al., 1996). In wild-type ovaries a dramatic increase in the levels of dE2F protein in follicle and nurse cell nuclei was observed at stage 10B, reflecting the induction of *dE2F* transcript (data not shown). dE2F protein was also detected in ovaries from the three female-sterile mutants, so neither the mutations in *dE2F* itself or in *dDP* affect dE2F protein stability (data not shown). We conclude that the temporal and spatial expression of *dE2F* RNA and of the dE2F protein match the developmental times at which defects occur in the *dE2F* and *dDP* mutants.

To confirm that *dE2F* expression correlates with E2F/DP transcription factor activity we examined the expression of E2F target genes in stage 10B egg chambers. In wild-type ovaries, *PCNA* and *RNR2* transcripts were observed at low levels during the early stages of oogenesis. However, both transcripts were induced at stage 10B (Fig. 5B). In contrast, the level of *PCNA* and *RNR2* transcripts at stage 10B was dramatically reduced in the dDP^{a1}, $dE2F^{i1}$, and $dE2F^{i2}$ female-sterile mutants (Fig. 5C and data not shown). In wild-type ovaries *cyclin E* expression is also induced at stage 10B, but the level of *cyclin E* transcripts was normal or slightly diminished in the mutant ovaries (data not shown). We tested for an effect of E2F on cyclin E by

Figure 5. *dE2F* expression correlates with E2F/DP activity.

Panel A is at a higher magnification than panels B and C.

(A) β-galactosidase staining of egg chambers from $dE2F^{rm729}/+$, a *dE2F* enhancer trap line (Brook et al., 1996; Duronio and O'Farrell, 1995). A stage 8-9 egg chamber is embedded between two stage 10B egg chambers. *dE2F* transcript (blue staining) is induced at stage 10B. This induction correlates with the transition to amplification in the follicle cells. The small blue cells are the follicle cells (arrowheads) and the larger cells are nurse cells (arrow).

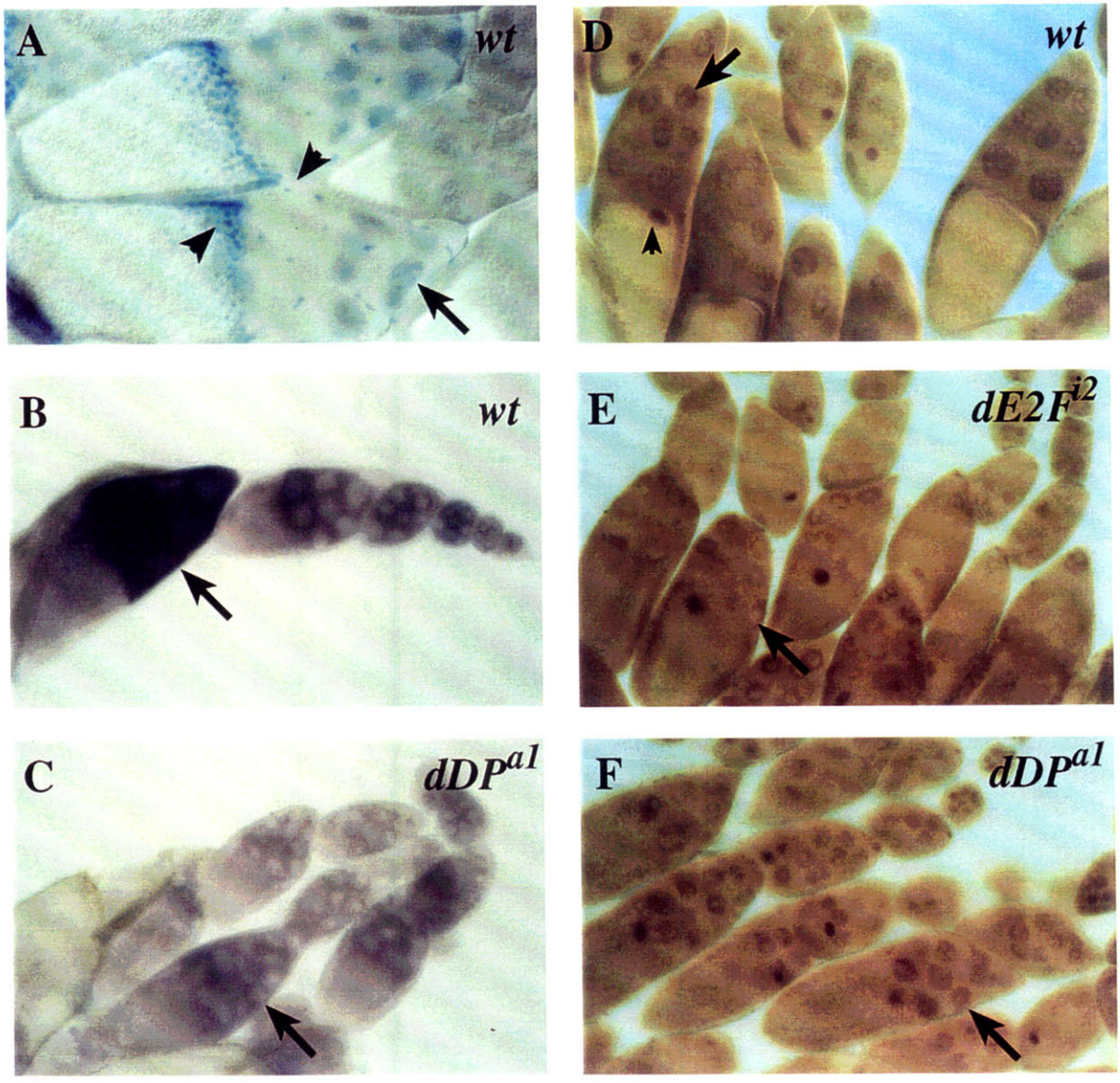
(B) *PCNA* expression in a continuum of developing control $dDP^{a1}/+$ egg chambers. *PCNA* transcripts were detected by whole-mount in situ hybridization. *PCNA* expression is seen in early egg chambers, but it is highly induced at stage 10B (arrow).

(C) In mutant dDP^{a1}/Df egg chambers *PCNA* expression is greatly diminished at stage 10B (arrow). The same effect on *PCNA* transcription was observed in both of the female-sterile *dE2F* mutants.

(D-F) Cyclin E protein levels in wild-type and mutant ovaries following staining with a monoclonal antibody (Richardson et al., 1995).

(D) A nurse cell nucleus (arrow) and the oocyte (arrowhead) are shown in a control $dDP^{a1}/+$ stage 10B egg chamber.

(E-F) The levels of cyclin E protein appear normal in mutant $dE2F^{i2}/Df$ and dDP^{a1}/Df egg chambers including stage 10B (arrows).


A
wt
D
wt
B
wt
E
dE2F^i2
C
dDP^a1
F
dDP^a1

monitoring the levels of cyclin E protein in wild-type and mutant ovaries with a monoclonal antibody against cyclin E (Richardson et al., 1995). In dDP^{a1}, dDP^{a2}/Df; *hsDP*/+, $dE2F^{i1}$ and $dE2F^{i2}$ mutants the levels of cyclin E protein were normal in nurse cells and follicle cells at all stages, including stage 10B (Fig. compare 5D to 5E,F). Thus, the induction of *PCNA* and *RNR2* transcripts is E2F dependent in the ovary while *cyclin E* appears to be dependent on the E2F transcription factor in some developmental contexts but not others.

dE2F and *dDP* promote nuclear envelope breakdown and apoptosis *in vivo*

Because ectopic *dE2F* expression drives apoptosis (Asano et al., 1996), we tested whether the female-sterile *dDP* and *dE2F* mutations block apoptosis. Nurse cells become polyploid, transfer their contents to the developing oocyte, and die by apoptosis (Foley and Cooley, 1998). The transport of nurse cell contents and subsequent cell death are linked by their requirement for cell death machinery. Drosophila caspase *dcp-1*, an essential cell death gene, inhibits the breakdown of the nurse cell nuclear envelope, and consequently the transport of contents from the nurse cells to the oocyte (McCall and Steller, 1998). The dramatic increase of dE2F protein in nurse cells at stage 10B anticipates the transfer of nurse cell cytoplasm to the developing oocyte. By stage 11, nurse cell nuclei have become permeable and their contents have diffused into the cytoplasm and are rapidly transferred to the oocyte via cytoplasmic bridges.

In the *dDP* and *dE2F* mutants, the nurse cells persisted well beyond the stage during which they are normally lost, suggesting a defect in nurse cell apoptosis. In addition, the oocytes were small, indicating a possible block to the transport of large amounts of RNA and protein from the nurse cells. Cyclin E and dE2F, proteins that localize to nurse cell nuclei, failed to diffuse into the cytoplasm even as late as stage 14 (data not shown). We tested whether the nurse cell nuclei become permeable at

stage 11 or later stages in the *dE2F* and *dDP* mutants by antibody staining of nuclear lamin. In the early egg chambers the nuclear envelope was intact in both the controls and mutants (Fig. 6A,B). In the dDP^{a1}, $dE2F^{i1}$, and $dE2F^{i2}$ mutants, however, the lamin staining persisted beyond the stage when the envelope is broken down in controls (Fig. 6D-F). The retention of nuclear contents by the mutants, therefore, resulted from defective nuclear envelope breakdown. We conclude that the activity of E2F is essential for the breakdown of lamins.

To determine directly whether *dDP* and *dE2F* are required for programmed cell death, we used the TUNEL method for *in situ* detection of dying nuclei. In wild-type and heterozygous control ovaries, all 15 nurse cell nuclei stained positively for TUNEL during stages 12-13 of oogenesis (Fig. 7A). By stage 14, the nurse cells degenerated, and TUNEL staining was no longer observed (Fig. 7D). In the *dDP* and *dE2F* mutants, however, a cluster of nurse cells remained attached to the stage 14 oocyte and failed to label with TUNEL (Fig. 7B,B',E,E'). Although apoptosis did not occur at the correct stage in *dE2F* and *dDP* mutants, in some egg chambers the persisting nuclei did stain with TUNEL at a later period (Fig. 7E). This indicated that apoptosis could eventually occur but was delayed. The results obtained by acridine-orange staining were consistent with those from the TUNEL analysis (data not shown). The effect on apoptosis was the same for all the female-sterile mutants: dDP^{a1}, dDP^{a2} */ Df; hsDP/+,* $dE2F^{i1}$, and $dE2F^{i2}$. Thus, E2F/DP activity and the C-terminal region of the *dE2F* gene promote normal apoptosis.

The *dE2F* and *dDP* mutants also allowed us to examine whether E2F/DP regulates the expression of apoptotic target genes. *Reaper* is an ideal candidate since it is transcriptionally regulated and induced prior to the onset of apoptosis (White et al., 1994). During oogenesis, *reaper* transcript was first observed during stage 9, but there was a dramatic induction of *reaper* at stage 10B (Fig. 7C) (Foley and Cooley, 1998; Nordstrom et al., 1996). *Reaper* continues to be expressed at high levels

throughout oogenesis. The induction of *reaper* is coincident with the induction of *dE2F*. *Reaper* transcript is reduced in the *dE2F* and *dDP* mutants (Fig. 7F). Even though *reaper* itself appears dispensable for nurse cell apoptosis (Foley and Cooley, 1998), its decrease in the mutants suggests that E2F/DP may regulate apoptotic genes.

Figure 6. E2F/DP is required for nuclear envelope breakdown.

Early and late egg chambers were stained with a monoclonal antibody to nuclear lamin Dm_0. The large rings surround the highly polyploid nurse cell nuclei and the small rings surround the follicle cells. In the early stages, nuclear envelope staining is identical for the sibling control (A) and the $dE2F^{i2}/Df$ mutant egg chambers (B). At stage 12, nurse cell nuclear envelope staining becomes diffuse in the controls (C), but the nuclear envelopes (arrow) persist in the $dE2F^{i2}/Df$ mutant (D). Nuclear lamin continues to be visible in dDP^{a1}/Df (E) and $dE2F^{i2}/Df$ (F) mutant nurse cells even at stage 14 (arrows). The same phenotype was observed for the $dE2F^{i1}/dE2F^{91}$ mutant.

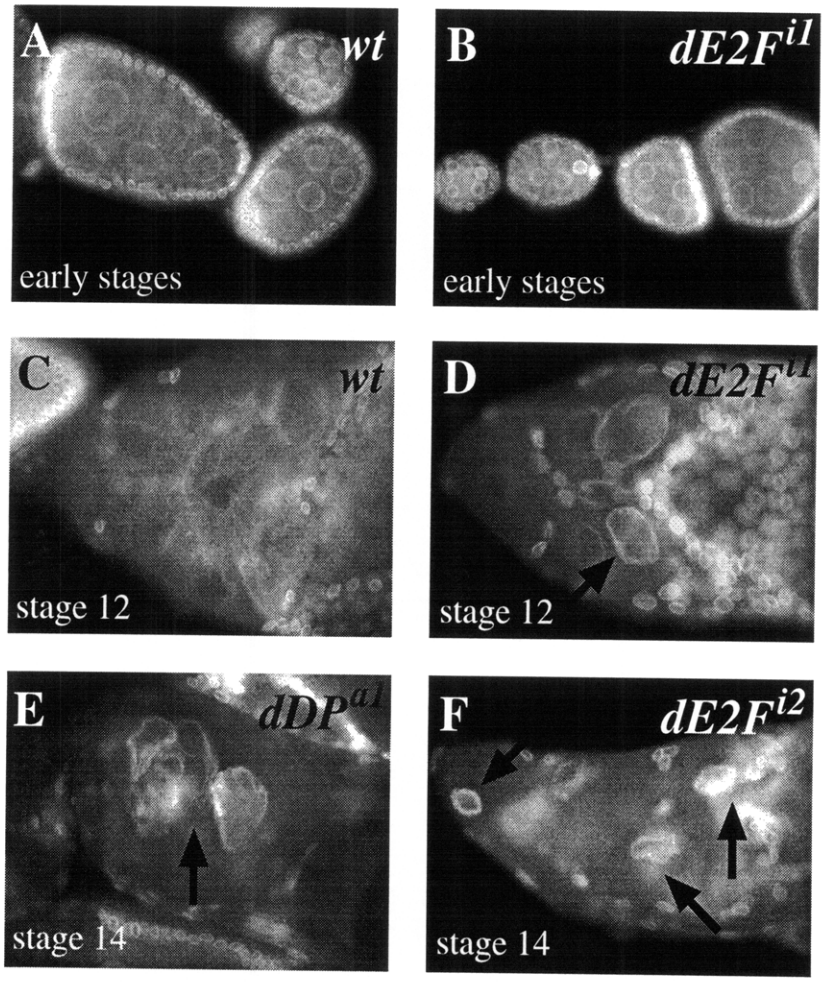

A
wt
early stages
B
dE2F^i1
early stages
C
wt
stage 12
D
dE2F^i1
stage 12
E
dDP^a1
stage 14
F
dE2F^i2
stage 14

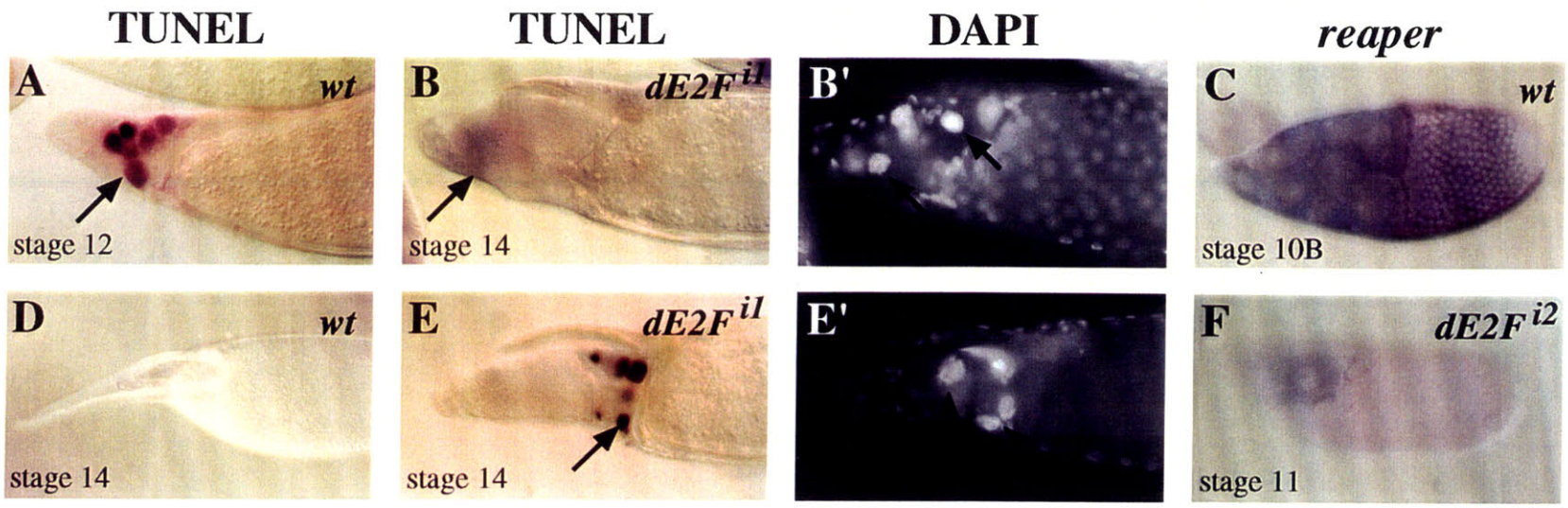
Figure 7. E2F/DP is necessary for normal apoptosis.

The TUNEL method was used for in situ detection of DNA fragmentation in dying nuclei. A DAPI counterstain was used to detect all nuclei.

(A) In wild-type ovaries nurse cell nuclei stain positively for TUNEL (purple staining and arrow) at stage 12. By stage 14 (D) the nurse cells are completely eliminated. In stage 14 $dE2F^{i1}/dE2F^{7172}$ mutant egg chambers (B and E) the nurse cell cluster remains attached to the oocyte. DNA fragmentation as evidenced by TUNEL staining does not begin (B) or is delayed (E). Failure to initiate apoptosis at the normal developmental time is also evidenced by the presence of nurse cell nuclei (arrows) in B' and E' , the DAPI counterparts to B and E, respectively. The same apoptotic defects were observed for dDP^{a1}/Df and $dE2F^{i2}/Df$ mutants.

(C) *Reaper* expression (purple staining) in a stage 10B control $dE2F^{i2}/+$ egg chamber. *Reaper* transcripts were detected by whole-mount in situ hybridization. The transcript is first observed at low levels at stage 9, but it is greatly increased in the nurse cells and follicle cells at stage 10B and in the later stages.

(F) Reduced reaper expression is shown in a stage 11 mutant $dE2F^{i2}/Df$ egg chamber. The level of expression is comparable to that seen in wild-type stage 9 egg chambers.

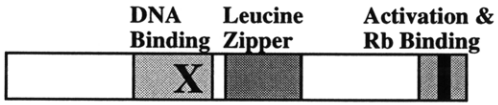
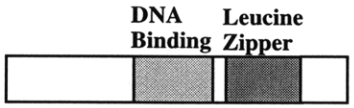
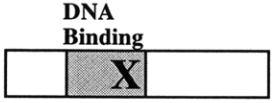

TUNEL
TUNEL
DAPI
reaper
A
wt
stage 12
B
dE2F i1
stage 14
B'
C
wt
stage 10B
D
wt
stage 14
E
dE2F il
stage 14
E'
F
dE2F i2
stage 11

Discussion

The mutations isolated in *dE2F* and *dDP* demonstrate an effect on nurse cell apoptosis and three examples in which E2F activity influences the activation of DNA replication origins within S phase (Table 2). First, in all three mutants the genomic intervals normally underreplicated during the endo cycle were found to be present at higher levels in the nurse cells. Thus fully functional E2F is required to limit replication within heterochromatic regions. Second, at a time when all the follicle cells would normally be undergoing origin firing in only four amplifying regions, in the *dDP* mutant the total genome incorporates BrdU. This reveals that E2F is needed to block general genomic replication origins during stages of egg chamber development when amplification occurs. Finally, E2F activity influences the extent to which amplifying regions of the genome replicate. The mutations in *dE2F* and *dDP* that should affect DNA binding block amplification, demonstrating a need for E2F in origin activation. The truncated form of dE2F, however, permits increased amplification. This indicates that E2F acts both to activate amplification origins and to restrict the number of times they fire.

In different developmental contexts we find that E2F activity is necessary either to inactivate or to activate origins. In mammalian systems E2F has been observed to act as a repressor when complexed with a pocket protein, but as an activator when free. Thus one possibility is that the opposite roles observed for E2F within S phase reflect differing complexes in which E2F resides at differing developmental points. It is striking that increased levels of amplification occur with a truncated form of dE2F lacking the RB-binding domain and transcriptional activator domain. Thus restriction of the extent of amplification origin use may be due to E2F complexed with RB and acting as a repressor. In contrast, the shut off of follicle cell genomic replication does not appear to require the RB interaction. E2F's action in restricting heterochromatin replication, however, could result from its repressor

Table 2. Summary of *dE2F*⁻ and *dDP*⁻ defects

Genotypes	Mutant Proteins	Viability	Viability with Reduced Cyc E dose	Apoptosis	Under-Replication	Shut off of Genomic Replication	Amplification
dE2F^{91}		–	–[a]	ND	–	ND	ND
dE2F^{i1}		+	–	–	–	+	–/+
dE2F^{i2}		+	+	–	–	+	++
dDP^{a1}		+	ND	–	–	–	–

[a]These animals die earlier in development with a two fold reduction of the zygotic *cyclin E* contribution.

activity in an RB complex, and the mutant effect due to derepression. Another possibility is that the inappropriate replication arises from decreased ability of the mutant forms to activate transcription of genes that normally hinder replication.

How does E2F affect replication origins? We envisage two potential mechanisms. The first possibility is that E2F affects origins in cis, independent of its activity as a transcription factor. It has been demonstrated that RB, when complexed to E2F, is capable of recruiting histone deacetylase and thereby converting chromatin to a compacted state (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). This state is correlated with inaccessibility to transcription factors, and it is reasonable to propose that it would also hinder binding of replication factors. Thus in this model E2F in complex with RB would cause histone deacetylation in the vicinity of replication origins, leading to underreplication of the nurse cell heterochromatin. A similar mechanism could shut off amplification origins, and the inability of $dE2F^{i2}$ protein to bind RB would prevent shut off in this mutant. Alternatively, it has been demonstrated in mammalian cells that the inappropriate binding of E2F to DNA during S phase (due to a failure to interact with cyclin A) causes S phase arrest (Krek et al., 1995). Therefore, it is possible that an analogous mechanism could be used during Drosophila oogenesis. For example, E2F could remain bound in heterochromatin and block replication, and rebind to follicle cell DNA to cause a shut off of genomic replication.

A more extreme version of the "in cis" model is that E2F directly affects replication origins. While this model necessitates a previously unrecognized E2F activity, the ABF transcription factor is known to bind to yeast ARS sequences (Bell et al., 1993) and transcription factors play a role in viral origin activation (De Pamphilis, 1993). In addition, cyclin E protein accumulates at amplification sites in the follicle cell nuclei (Calvi et al., 1998). With polyclonal antibodies against dE2F (Asano et al., 1996) we were unable to detect dE2F at discrete nuclear foci when

amplification is occurring (I. Royzman and T. Orr-Weaver, unpublished observations). This result does not support this model, but it still remains a formal possibility.

An alternative is that E2F mediates its effects on replication indirectly, *in trans*, via its role as a transcriptional repressor and/or activator. Several of the genes regulated by E2F affect DNA replication origins. The transcription of at least one *MCM* gene, an *ORC* subunit, and *CDC6* is dependent on E2F (Duronio et al., 1998; Ohtani et al., 1996; Yan et al., 1998). Chorion gene amplification is diminished in a female-sterile mutation in *orc2* (Calvi et al., 1998; Landis et al., 1997). Thus E2F activity may be necessary to produce the proteins needed to establish prereplication complexes on origins.

In the "*in trans*" model additional explanations are required to account for the effect of mutations in *E2F* on origin activity in particular genomic regions. It is possible that some of the differential effects on replication may be explained by timing. In a weak allele of *cyclin E*, heterochromatic replication in the nurse cells is increased (Lilly and Spradling, 1996). To explain this, Lilly and Spradling proposed that reduced cyclin E activity leads to dampened oscillations in cyclin E protein during the nurse cell endo cycles, and this prolongs S phase. During the lengthened S phase sequences that replicate late in S phase could be replicated in the mutant. In the *dDP* and *dE2F* mutants, S phase does appear to be prolonged (Duronio et al. 1998; Royzman et al. 1997). While an explanation of lengthened S phase can account for the replication of nurse cell heterochromatin, it is hard to reconcile with the replication of the total genome in follicle cells in dDP^{a1}, and the failure of amplification in dDP^{a1} and $dE2F^{i1}$. However, even in the nurse cells it does not appear that the *dDP* and *dE2F* mutant effects are a consequence of reduced levels of cyclin E. We failed to detect a reduction in *cyclin E* transcripts or protein in the mutant ovaries. Therefore it may be that the *cyclin E* mutation decreases the amount of active E2F, altering the replication parameters.

In addition to the roles within S phase, the mutant phenotypes reveal that *dDP* and *dE2F* promote apoptosis in cells that are destined to die during the normal course of development. Although ectopic *dE2F* expression alone is sufficient to induce apoptosis (Asano et al., 1996), developmental cell death requires the function of the E2F/DP heterodimer. The observed defects in the *dDP* and *dE2F* mutants include inhibition or delay of DNA fragmentation, failure to breakdown nuclear lamins, and reduced expression of a pivotal cell death gene. The broad spectrum of apoptotic defects suggest that E2F/DP plays a regulatory function for the initiation of developmental cell death. These findings support and extend the previous observation that cultured thymocytes from *E2F-1*$^{-/-}$ mice are less susceptible to apoptosis.

The *dE2F* mutations described in this study have been particularly useful because they retain some activity. They permitted us to analyze the role of E2F in replication and apoptosis in a biological context, adult oogenesis, in which the parameters of S phase could be defined with precision using cell biology. The surprising contradiction between the molecular nature of the alleles and the weak phenotypes provides insights into the mechanisms by which E2F influences gene expression. The *dE2F*^{i2} allele is particularly striking because it lacks the activation and RB-binding domains. Yet, in contrast to the *dE2F* null (*dE2F*^{91}) the *dE2F*^{i2} mutation does not affect viability. The difference in the severity of phenotypes suggests that a previously unidentified activity in the N-terminus of *dE2F* is significant for transcriptional activation *in vivo*. The *dE2F*^{i1} and *dDP*^{a1} mutations are analogous, yet the two mutants do exhibit some differences in phenotype (Table 2). The *dDP* mutant fails to extinguish genomic replication in the follicle cells. Although it is possible that the two subunits of E2F have independent activities, we favor the interpretation that the absence of an effect on genomic replication in the

dE2F mutant is due to compensating dE2F protein from a second *dE2F* gene (D.S. Huen and N. Dyson, personal communication).

These alleles are relevant also to the activity requirements for E2F in apoptosis. Recent studies showed that overexpression of transactivation defective *E2F-1* mutants but not *E2F-1* DNA-binding mutants can induce apoptosis in cell culture (Hsieh et al., 1997; Phillips et al., 1997). Further, RB overexpression inhibits apoptosis induced by wild-type *E2F-1* and RB-binding competent *E2F-1* mutants (Hsieh et al., 1997). These observations led to the notion that DNA binding rather than the transactivation activity of E2F-1 is necessary for its apoptotic function, and that apoptosis is the result of alleviation of RB-E2F1 repression of apoptotic target genes. The phenotypes exhibited by the *dE2F* and *dDP* mutants do not support this model. We observed either an apoptotic block or delay in all the mutants tested, including $dE2F^{i2}$ which lacks the RB-binding domain. Thus, we favor a model in which the E2F/DP heterodimer is an activator of the apoptotic pathway.

The biological roles uncovered for E2F in this study highlight the importance of examining mutant loss-of-function phenotypes within a multicellular organism under different developmental contexts. The *dE2F* and *dDP* mutations were recovered due to a failure to activate E2F-dependent G1-S transcription. However, phenotypic analysis of the mutants has shown that the role of E2F/DP in S phase is not limited to the onset of DNA replication. The replication phenotypes observed in *dE2F* and *dDP* mutant adults reveal that E2F/DP controls differential regulation of replication origins within S phase. This study raises the intriguing possibility that the activity of transcription factors may regulate the timing and choice of replication origins.

Materials and methods

Fly strains

dDP^{a1} and *dDP*^{a2} alleles were described previously (Royzman et al., 1997). The deficiency uncovering *dDP*, *Df(2R)vg56*, was provided by R. Duronio (Duronio et al., 1998). *dE2F*^{7172}, *dE2F*^{91}, *dE2F*^{rm729} alleles (Duronio et al., 1995) and the transgenic lines *P[w*$^{+}$*, hsp70-dE2F]*, *P[w*$^{+}$*, hsp70-dDP]* were provided by N. Dyson (Duronio et al., 1996). The deficiency uncovering *dE2F*, *Df(3R)e-BS2* was obtained from the Bloomington Stock center. The *cyclin E*^{01672} allele was provided by A. Spradling (Lilly and Spradling, 1996). *Cyclin E*^{PZ5} was provided by J. Roote (University of Cambridge, UK).

New mutations in dE2F

dE2F^{i1} and *dE2F*^{i2} were isolated from third chromosome mutant lines established in the lab of R. Lehmann (Skirball Institute, New York University, Medical Center, NY). The EMS mutagenesis and crosses to establish balanced stocks have been described (Moore et al., 1998). We screened these lines for mutations that disrupt G1-S transcription by the strategy previously described (Royzman et al., 1997).

In order to demonstrates that *dE2F*^{i1} and *dE2F*^{i2} are mutations in the *dE2F* gene, both mutant lines were sequenced. Genomic DNA was isolated from adults transheterozygous for the mutagenized chromosome and *Df(3R)e-BS2*. The *dE2F* open reading frame was amplified from the mutant genomic DNA, and the PCR products were sequenced directly by Research Genetics.

Cytological analysis and microscopy

In situ hybridizations (Tautz and Pfeifle, 1989) were carried out with digoxigenin-labeled RNA probes exactly as described in Royzman et al. (1997). For whole-mount *in situ* hybridization to ovaries, ovaries were prepared as described by Ephrussi et al. (1991) and treated for 35 min in proteinase K. Subsequent steps were carried out as for embryos. The *reaper* cDNA was provided by H. Steller (White et al., 1994). The β-galactosidase expression pattern in $dE2F^{rm729}$ ovaries was determined according to a standard protocol (Montell et al., 1992). TUNEL and acridine orange staining were used to detect apoptotic cells in ovaries. The ovaries were processed as described in McCall and Steller (1998). Ovaries assayed for cell death by TUNEL were counter-stained for 20 min with 1ug/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma). In the *dE2F* and *dDP* mutants occasional degenerating early egg chambers (stage 8 and earlier) were observed.

Ovaries were labeled with BrdU as described previously (Lilly and Spradling, 1996). BrdU labeling was visualized using a goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad) at a dilution of 1:200. Nuclei were counter-stained with DAPI as above. For antibody stainings, ovaries were fixed in 8% EM grade formaldehyde (Ted Pella) for 5 min, and extracted for 2 hours in 1% Triton X-100 in PBS, and then blocked for 1 hour in 1XPBS, 1% BSA, .3% Triton X-100, and 2% normal goat serum. For detection of cyclin E, a mouse monoclonal antibody 8B10 was provided by H. Richardson and used at a dilution of 1:5 (Lilly and Spradling, 1996; Richardson et al., 1995). The HRP goat anti-mouse secondary antibody (Bio-Rad) was used at 1:200. For E2F detection, a rabbit polyclonal antibody was provided by R. Wharton (Asano et al., 1996) and used at a dilution of 1:100. We showed that this antibody specifically recognizes dE2F in the ovary, because $dE2F^{91}/Df$ females rescued to the adult stage by *hsE2F* did not show protein in the ovary. An HRP-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research

Laboratories) was used at a dilution of 1:100. Nuclear lamin Dm_0 staining was carried out using Ab101, an antibody provided by K. McCall and H. Steller (Smith et al., 1987). Ab101 was diluted 1:1.5 and detected with a Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immuno Research Laboratories) at a dilution of 1:150. The staining pattern with this antibody has been described (McCall and Steller, 1998) and is similar to that seen with a polyclonal antibody to nuclear lamin Dm_0 (Smith and Fisher, 1989).

A Zeiss Axiophot microscope equipped with Nomarski optics and fluorescence was used to examine and photograph the embryos and ovaries. Plan-Neofluar 10x, 20x and 40x objectives were used.

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Afterword

The roles for *dE2F* and *dDP* uncovered in this study provide significant inroads into understanding the biology of E2F. They also raise mechanistic questions, some of which can be addressed biochemically. In addition, the *dDP* and *dE2F* mutations can be used as tools to identify interacting genes and further explore the regulation of G1-S progression and differential replication. Moreover, there are a number of phenotypes associated with the *dDP* adult viable allele that have not yet been explored: more female flies than males flies are recovered, the males are sterile, female meiosis is defective. The differential effect on female and male viability is very interesting because it suggests that *dDP* may have a role in sex determination in addition to its role in S phase. Genetic approaches can be taken to evaluate this possibility. In addition to the mutations in *dDP* and *dE2F*, the *in situ* hybridization screen has also led to the identification of a number of novel genes. These new genes open yet other experimental directions.

Null mutations in *dE2F* and *dDP* cause lethality late in development with some tissues being underdeveloped or absent. Although lethality is at the larval/pupal boundary, a pronounced effect on E2F-dependent G1-S transcription was observed in eight hour embryos. It is clear that the bursts of *cyclin E*, *PCNA* and *RNR2* transcription that normally precede S phase are not essential for the G1-S transition. We postulate that in the mutants the cell cycle could be driven by basal levels of transcripts, maternal pools of replication machinery, and post-transcriptional regulation. Late in development the transcriptional activity appears to be needed, since the mutants die with tissues that are not fully developed. The $dE2F^{i2}$ mutant, therefore, came as a surprise. This mutant completely lacks the transcriptional activation domain of dE2F, but viability is not affected. Thus, the bursts of E2F-dependent G1-S transcription appear not to be needed even in late development.

The difference in the severity of phenotype between the *dE2F*^{i2} mutation and the null is striking but difficult to reconcile with all that we know about the E2F protein from studies using mammalian cells. dE2F^{i2} retains significant physiological activity that cannot be explained simply by lack of RB-mediated repression, since a null also lacks RB-mediated repression. The possible explanations seem to be the following: (1) a previously unidentified activity in the N-terminus of dE2F is significant for transcriptional activation *in vivo* (although the bursts of S phase transcription are not observed by *in situ* hybridization in the *dE2F*^{i2} embryo or adult); (2) dE2F transcriptional activation is not essential for viability but instead DNA bound dE2F is used as a docking site by other molecules to regulate chromatin structure or in some other way to affect transcription. These possibilities are difficult to address experimentally. It is feasible to create different mutant form of *dE2F* and to put them back into the fly. It would be very striking if a region of dE2F that can only dimerize with dDP and bind DNA could rescue the *dE2F* null. The other way to try to understand what dE2F is actually doing is to take a genetic approach and to isolate functions that depress the activity of dE2F^{i2}. For example, one could look for enhancers that would change the lethal phase of the *dE2F*^{i2} mutant from the adult to a pupal stage. This is a long haul but is likely to ultimately provide significant insights into the activity of dE2F.

The increased levels of amplification in the *dE2F*^{i2} mutant as compared to wild type suggest that E2F/DP may affect origins independently of its role as a transcriptional activator of S phase genes. Further experiments are needed to evaluate this possibility. The cis-acting elements involved in chorion gene amplification have been identified. Biochemical approaches can be taken to determine whether E2F/DP is bound in the vicinity of these elements. A specific interaction of E2F/DP with these cis-acting elements would provide support for a model where E2F/DP affects the activity of DNA replication origins "in cis" rather

than through the transcriptional activation or repression of other proteins. In the best scenario, the biochemistry would be confirmed by cytology. I did not see dE2F localize to subnuclear foci with a polyclonal dE2F antibody (see Chapter 4). However, possibly a monoclonal antibody against dE2F may reveal subnuclear dE2F localization, and the identity of the subnuclear foci can be verified with anti-dE2F and anti-BrdU double labeling.

A number of genetic screens can be initiated to identify genes that regulate the G1-S transition and differential replication. The *dE2F* and *dDP* adult viable mutants could be used as a starting point to obtain genes that are involved in the same biological process. It should be possible to alter the mutant phenotype of interest due to a twofold reduction in the zygotic contribution of an interacting gene. For example, one could look for dominant suppressers or enhancers that alter the lethal phase of the dDP^{a1} mutation from reduced viability to full viability or pupal lethality. The interacting genes may be regulators of the G1-S transition or essential replication genes. A possible added bonus is that suppressers or enhancers of the differential effect on male and female viability in the dDP^{a1} mutant may also be recovered. These may turn out to be known regulators of sex determination and would support a role for *dDP* in the sex determination pathway.

Two possible screens could be carried out to identify genes involved in differential replication control: (1) a screen for suppressers of the over-replication phenotype in mutant *dDP* or *dE2F* nurse cell nuclei; (2) a screen for suppressers of the chorion amplification defect in the dDP^{a1} or $dE2F^{i1}$ mutants. The first screen is somewhat labor-intensive because it involves dissecting ovaries and DAPI staining. The second screen is more attractive because it is simpler; it is very easy to discern a change from thin eggshells in the non-amplifying mutants to the normal white eggshells. The identification of gene products that interact with E2F/DP may help to

determine how E2F/DP acts to positively and negatively regulate origin activity during S phase.

It is possible to characterize and clone the other mutations identified in the *in situ* hybridization screen and discussed at the end of Chapter 2. These mutations have highly specific effects on S phase reporter genes and may provide considerable insight into the control of DNA replication. *l(2)51Ec* is especially interesting because it appears to be required both for S phase entry as well as the completion of S phase. Because the screen has been successful on the second chromosome and the technique for carrying out *in situ* hybridizations on multiple lines has been perfected to allow relatively rapid screening, it would be very interesting to extend the screen to the X chromosome. A comprehensive screen on the third chromosome is also worth doing, since only a 1000 mutagenized lines have been examined. This approach may lead to the identification of a regulator that is specific to the endo cycle, and certainly additional S phase regulators that are shared by mitotic and endo cycle tissues would be isolated.

The phenotypes associated with the adult viable *dDP* and *dE2F* mutations really support the approaches discussed in the appendix for identifying hypomorphic mutations in genes important for endo cycles. The *dDP* and *dE2F* mutants meet all the phenotypic criteria that I used to select putative endo cycle regulators: reduced body size, bristle defects, and female sterility. *diminutive*, Drosophila *myc*, also meets the same criteria. In contrast to my expectations when I first initiated the EMS screen for small adults and ordered existing small fly mutants (see Appendix), mutations in genes that promote endoreplication (*dDP*, *dE2F* and *dmyc*) do not cause a dramatic reduction in the level of nurse cell polyploidy. I think with this observation in mind, it would be worth examining existing mutations that cause small adults.

Additionally, the relationship of *dE2F* and *dDP* with *dmyc* could be explored. The phenotypes of the hypomorphic *dDP*, *dE2F*, and *dmyc* alleles are very similar;

the effects on bristles are identical. Moreover, *dmyc* and *dmax* transcripts are expressed in the pattern of S phase reporter genes and DNA synthesis in late embryos (Gallant, et al. 1996); the expression in the CNS is relatively low but it is quite robust in the endodomains. It is likely that both transcripts are E2F/DP dependent. This can be readily determined by examining *dmyc* and *dmax* transcripts in the background of an *dE2F* null or *dDP* null mutant. It would be also interesting to look for a genetic interaction between *dE2F* (or *dDP*) and *dmyc*. The ovaries form *dmyc* mutant females can also be examined by DAPI staining for effects on late replication of heterochromatin. I did not specifically look for this phenotype in my initial characterization of this mutant.

The identification of gene products involved in the G1-S transition and differential replication will enable us to understand the molecular mechanisms that control these processes and the role of E2F/DP.

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Appendix

Using small-fly mutants to identify regulators of the endo cycle

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The endo cycle begins during Drosophila embryogenesis and consists of alternating DNA synthesis and gap phases (Smith and Orr-Weaver 1991). This modified cell cycle leads to the production of polyploid and polytene cells. Drosophila larvae use polytenization rather than proliferation as the primary mechanism of larval growth. The switch from a mitotic cycle to the endo cycle is a recurrent theme in Drosophila development. In addition to the switch in embryogenesis, bristle precursor cells within the pupal epidermis, and the nurse and follicle cells of the adult ovary become polyploid.

My studies of the regulation of the endo cycle began with a screen for mutations that cause small adults. The hypothesis underlying this approach was that since larval growth is achieved by increasing the ploidy of larval cells, a partial block to the endo cycle should result in small larvae and small pupae which give rise to small adults. In addition, compromised ability to undergo endo cell cycles is expected to cause female sterility and bristle defects. Females homozygous for a hypomorphic mutation should be sterile due to deficient polyploidization of the nurse cells. Bristle shafts and sockets were expected to be shorter and smaller respectively since the bristle precursor cells (trichogen and tormogen cells) undergo endo cycles (Lees and Waddington 1942). More severe phenotypes were expected when the mutation is put over a deficiency.

We suspected that both cell cycle and nutritional mutants could give rise to a small adult phenotype. Mosaic analysis would be used to differentiate between these two classes of mutants. Endo cycle regulator mutations, unlike nutritional mutations, were expected to have an immediate effect in a homozygous clone. The effect on nurse cells and bristles could then be examined soon after the induction of a homozygous clone.

I carried out an F1 screen for EMS induced mutations on the X chromosome that cause small adult males (Fig. 1). Isogenized *yw* males were fed 35 mM EMS and

mated to virgin females from a virginator stock (*C(1)M4, y/ shi^{ts}/y^{+}Y*). The virginator stock eliminated the need to collect virgins in the parental cross because males die at 29°C due to the *shi^{ts}* X chromosome. In addition, a recessive mutation on the X chromosome could be seen in the F1 generation in the male progeny. I made sure to avoid over-crowding of the mutant progeny to avoid false positives due to environmental conditions. Also, because small adults are known to eclose later than wild type, I collected the F1 progeny both early and late in order not to miss the males that eclose a few days late. 5385 females and 2433 males were recovered from the parental cross. The mutagenesis, therefore, caused approximately 55% sex-linked lethals. Of the 2433 males, 13 putatives were retested. 6 of the small males were sterile, and the other 7 did not breed true.

I decided not to pursue the F1 screen further due to two problems with this approach. It was not clear at the time the degree to which a weak mutation in an endo cycle regulator would affect the size of the flies. In addition, EMS causes a high frequency of mosaicism (Ashburner 1989). This makes it difficult to recover mutations from an F1 screen.

In addition to the F1 screen for small males, I examined a number of already existing mutants that have defects in tissues which undergo endo cycles. The most promising candidates were *diminutive*, *tiny*, and *minus*, because the mutant adults were small, female sterile, male fertile, and had reduced bristles. *diminutive* is a hypomorphic mutation with a striking bristle phenotype that becomes more severe over deficiency (Fig. 2). In addition, the tergites are etched; this is consistent with a role for *diminutive* in the mitotic cell cycle as well (Fig. 2). Therefore, *diminutive* is

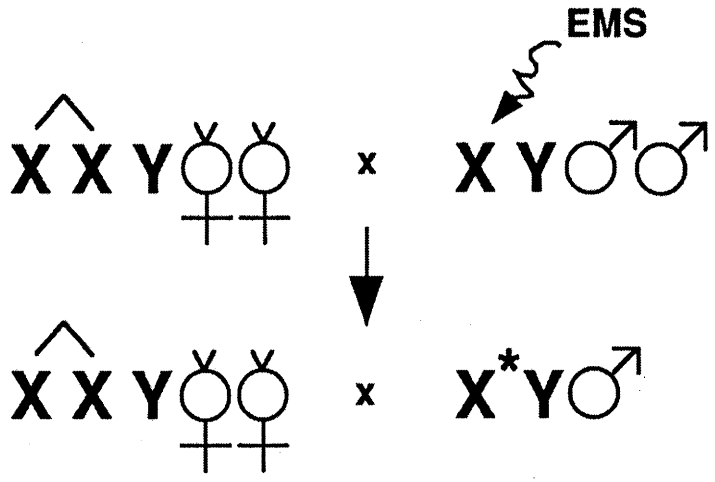

Fig. 1 F1 screen for mutations on the X chromosome that cause small adults.

Isogenized *yw* males were mated to *C(1)M4, y / shi^{ts} / y^{+} Y* virgin females. 2433 mutant males were examined from the parental cross. 13 putative small males were mated singly to *C(1)M4, y / shi^{ts} / y^{+}Y* virgins and retested. 6 of the males were sterile and the other 7 not breed true.

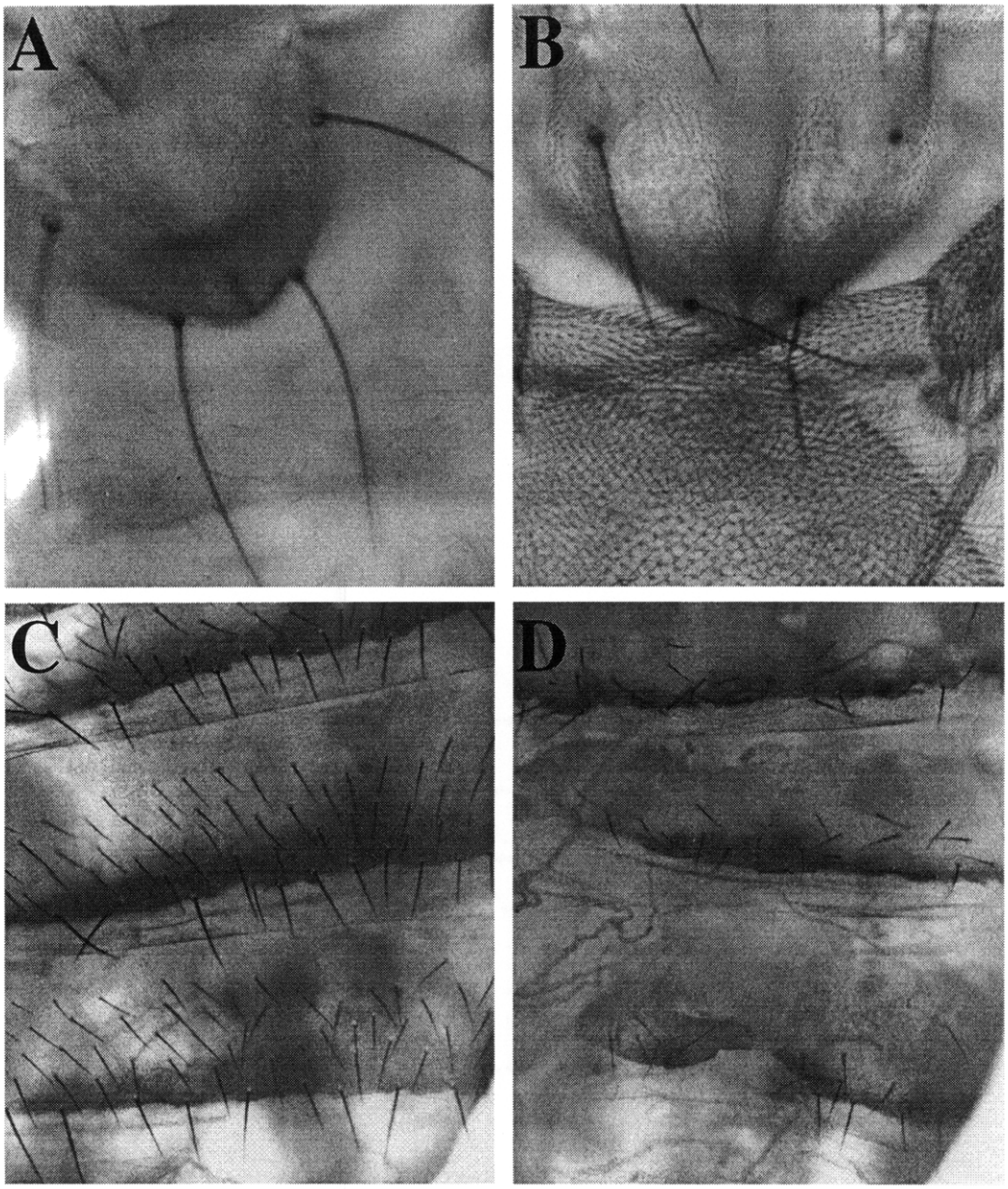

Figure 2. Bristle defects in the *dm* mutant.

(A) *dm*/*dm* scutellar bristles are more thin and reduced as compared to wild-type bristles.

(B) The mutant phenotype is even stronger over the deficiency. *dm*/*Df* scutellar bristles are very thin and short, one is missing.

(C) *dm*/+ bristles and tergites.

(D) *dm*/*Df* bristles are reduced in number and in size and the tergites are etched.

not a specific regulator of the endo cycle. The nurse cell phenotype as revealed by the DNA dye DAPI was not pronounced, and I did not pursue *diminutive* further. *tiny* was also not pursued because cytological examination of the mutant ovaries did not reveal a decrease in the levels of polyploidy in nurse cells and follicle cells. Interestingly, it has been shown recently that *diminutive* encodes a Drosophila homologue to the mammalian oncogene *myc* (Gallant, et al. 1996; Schreiber-Agus, et al. 1997).

DAPI staining of *minus* mutant ovaries revealed a distinctive phenotype. Normally, nurse cell endoreplication results in polytene chromosomes during the early stages of oogenesis (prior to stage 4). However, at stage 4 the polytene nurse cell chromosomes dissociate, so the nurse cell nuclei become polyploid (Spradling 1993). The nurse cells continue to undergo endo cycles but the nuclei remain polyploid for the remainder of oogenesis. The transition from polyteny to polyploidy is not understood. It is thought that separation of polytene chromosomes may involve chromatin condensation and therefore may require some mitotic activities. In the *minus* mutant the nurse cells are able to undergo endo cycles but the normal progression of chromatin reorganization is defective; condensed chromosomes can be seen even at late stages of oogenesis (Fig. 3). In addition, *minus* mutant mothers can occasionally lay eggs. These eggs do not develop, and DAPI staining of the eggs revealed that they are arrested during the early embryonic divisions with 8-32 nuclei. It is possible that *minus* provides an essential mitotic function in embryos and at the transition from polyteny to polyploidy in the ovary.

I mapped *minus* extensively using deficiencies in region 59. The position of *minus* relative to these deficiencies and other genes in the 59 region is included in the thesis of Dan Moore (Moore 1997). These same deficiencies were used by Sharon Bickel to define the *ord* gene molecularly (Bickel, et al. 1996), and it seemed likely

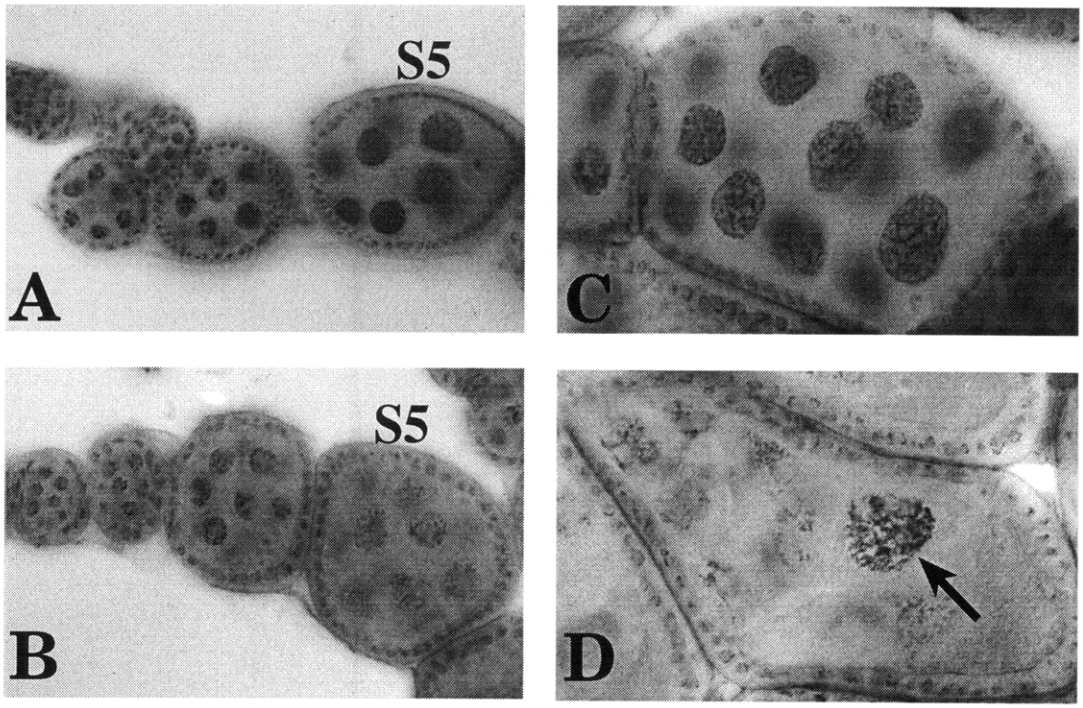

Figure 3. The oogenesis phenotype of *minus*. A basic fuchsin stain was used to visualize DNA in egg chambers from wild-type (A,C) and *minus* mutant females (B,D).

(A) In wild-type egg chambers the nurse cell nuclei are polytene prior to stage 4; the chromosomes become bulbous at stage 4 and by stage 5 (S5) the nurse cell nuclei appear polyploid rather than polytene.

(B) In contrast, in *minus* mutant egg chambers the transition from polytene to polyploid nurse cell nuclei is defective. The chromosomes appear polytene at stage five.

(C) A late wild-type egg chamber is shown. The 15 nurse cell nuclei are polyploid.

(D) In late *minus* mutant egg chambers the nurse cell nuclei have increased in size but the chromatin remains condensed (arrow).

that *minus* is contained in the molecular walk for *ord*. I felt that a molecular analysis of *minus* would not yield insight into the regulation of S phase or the switch from a mitotic cycle to an endo cycle. Consequently, I did not pursue the phenotypic or molecular characterization of *minus* further. Instead, I embarked on a genetic screen to identify regulators of S phase in mitotic and polytene cells in the embryo.

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